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**PCT**

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<p>(21) International Application Number: <b>PCT/US98/02377</b> (22) International Filing Date: <b>5 February 1998 (05.02.98)</b> (30) Priority Data: <b>08/795,876 6 February 1997 (06.02.97) US</b> (71) Applicant: <b>CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US).</b> (72) Inventors: <b>GERSHENGORN, Marvin, C.; 400 East 56th Street, New York, NY 10022 (US). GERAS-RAAKA, Elizabeth; 34 Maplewood Avenue, Dobbs Ferry, NY 10522 (US). NUSSENZVEIG, Daniel, R.; 50 Park Avenue, New York, NY 10016 (US).</b> (74) Agents: <b>WEYAND, Karla, M. et al.; Nixon, Hargrave, Devans &amp; Doyle LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).</b></p>		<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>With international search report.</i></p>
<p>(54) Title: <b>LIBRARY SCREENING AS A STRATEGY TO CLONE DRUGS FOR G PROTEIN COUPLED RECEPTORS</b></p> <p>(57) Abstract</p> <p>The present invention is directed to a strategy to identify small peptides that activate any G protein coupled receptor (GPCR) or inactive any constitutively active GPCR by screening combinatorial peptide libraries. The invention comprises expressing a peptide of a peptide library tethered to a GPCR of interest in a cell, and monitoring the cell to determine whether the peptide is an agonist or negative antagonist of the GPCR of interest. The peptide is tethered to the GPCR by replacing the amino terminus of the GPCR with the amino terminus of a self-activating receptor, and replacing the natural peptide ligand present in the amino terminus with the library peptide. In one embodiment for discovery of agonists, a ligand of the self-activating receptor is used to cleave the resulting amino terminus to expose the peptide of the peptide library. In another embodiment for discovery of agonists or negative antagonists, the GPCR construct ends in the peptide so the peptide is always exposed. Preferably, the self-activating receptor is the thrombin receptor and the ligand of the self-activating receptor is thrombin.</p>		

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## LIBRARY SCREENING AS A STRATEGY TO CLONE DRUGS FOR G PROTEIN COUPLED RECEPTORS

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10                           **FIELD OF THE INVENTION**

The present invention relates to drug discovery, and more particularly to a strategy to clone drugs for G protein coupled receptors.

15                           **BACKGROUND OF THE INVENTION**

Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description. The disclosures of these publications in their entireties are hereby incorporated by reference in this application.

20           It has been estimated that more than 50% of the drugs in clinical use today are directed at G protein coupled receptors (GPCRs). Small peptides can activate a number of receptors of this family, such as receptors for thyrotropin-releasing hormone (TRH), which is a tripeptide (Gershengorn and Osman 1996), thrombin, for which a hexapeptide is a full agonist (Tapparelli et al. 1993), and formyl-Met-Leu-Phe, which is a tetrapeptide (Perez et al. 1994). Small molecules can inactivate constitutively active GPCRs, such as benzodiazepines, which inactivate TRH receptor mutants that are constitutively active (Heinflink et al. 1995) (a constitutively active receptor is one that signals in the absence of agonist).

35           It appears that these small molecules interact primarily, if not exclusively, with the transmembrane (TM) bundle or extracellular (EC) loops of GPCRs (Cascieri et al. 1995). For example, it

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appears that the "activation domain" of a GPCR with a large EC amino terminus, such as the receptor for calcitonin, is present within the region of the receptor from the beginning of TM helix one to the C-terminus, which includes the TM bundle and EC loops (Stroop et al. 1995).

The discovery of peptides that could activate GPCRs or inactivate constitutively active GPCRs may have enormous potential for clinical applications because a number of peptide agonists of GPCRs are currently used therapeutically and diagnostically. In the shorter term, the discovery of such peptides will yield reagents that could be used by pharmaceutical companies to identify ligands for or functions of "orphan" receptors.

#### SUMMARY OF THE INVENTION

To this end, it is an object of the subject invention to provide a strategy to discover small peptides that will activate any G protein-coupled receptor (GPCR) or inactivate any constitutively active GPCR. These peptides could serve as lead chemicals for design of clinically useful drugs or could be used to identify the natural ligand or physiologic function of "orphan" receptors, that is, putative receptors that have been identified (i.e., cloned) but for which the function is unknown. The strategy uses combinatorial peptide libraries tethered to the GPCR. With this approach, millions of random peptides of a given length can be tested for activity in the context of a library and those that activate GPCRs or inactivate constitutively active GPCRs can be identified.

The invention thus provides a method of identifying peptide agonists or negative antagonists of a G protein coupled receptor of interest. The method comprises expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in

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a cell, and monitoring the cell to determine whether the peptide is an agonist or negative antagonist of the G protein coupled receptor of interest.

In one embodiment for identifying peptide agonists, the expression of a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises preparing a G protein coupled receptor construct, introducing the G protein coupled receptor construct into a cell, allowing the cell to express the G protein coupled receptor encoded thereby, and exposing the cell to a ligand of a self-activating receptor, wherein the ligand cleaves the G protein coupled receptor construct so as to expose the inserted peptide of the peptide library. The G protein coupled receptor construct for identifying a peptide agonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which is a peptide agonist for activating the self-activating receptor; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

In a further embodiment for identifying peptide negative antagonists, the G protein coupled receptor of interest is a constitutively active G protein coupled receptor and the expression of a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises preparing a constitutively active G protein coupled receptor construct, introducing the constitutively active G protein coupled receptor construct into a

cell, and allowing the cell to express the  
constitutively active G protein coupled receptor  
encoded thereby. The constitutively active G protein  
coupled receptor construct for identifying a peptide  
5 negative antagonist, which is also provided by the  
subject invention, comprises a nucleic acid molecule  
encoding a constitutively active G protein coupled  
receptor with a deleted first amino terminus; a nucleic  
acid molecule encoding a second amino terminus of a  
10 self-activating receptor attached to the nucleic acid  
molecule encoding the constitutively active G protein  
coupled receptor at the deleted first amino terminus,  
the second amino terminus having a deleted portion  
which includes a peptide agonist for activating the  
15 self-activating receptor as well as any amino acids  
positioned amino terminally to the peptide agonist; and  
a nucleic acid molecule encoding the peptide of the  
peptide library inserted into the second amino terminus  
and replacing the deleted portion.

20 In a still further embodiment for identifying  
peptide agonists, the expression of a peptide of a  
peptide library tethered to a G protein coupled  
receptor of interest in a cell comprises preparing a G  
protein coupled receptor construct, introducing the G  
25 protein coupled receptor construct into a cell, and  
allowing the cell to express the G protein coupled  
receptor encoded thereby. The G protein coupled  
receptor construct for identifying a peptide agonist,  
which is also provided by the subject invention,  
30 comprises a nucleic acid molecule encoding a G protein  
coupled receptor with a deleted first amino terminus; a  
nucleic acid molecule encoding a second amino terminus  
of a self-activating receptor attached to the nucleic  
acid molecule encoding the G protein coupled receptor  
35 at the deleted first amino terminus, the second amino  
terminus having a deleted portion which includes a  
peptide agonist for activating the self-activating

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receptor as well as any amino acids positioned amino terminally to the peptide agonist; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

#### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1 is a diagram of a G protein coupled receptor;

Fig. 2 is a diagram of a thrombin receptor;

Fig. 3 is a diagram of a peptide of a peptide library;

Fig. 4 is a diagram of a G protein coupled receptor construct according to the subject invention;

Fig. 5 is a diagram of a constitutively active G protein coupled receptor construct according to the subject invention;

Fig. 6 is a diagram of the putative two-dimensional topology of the human calcitonin receptor;

Fig. 7 is a diagram of the putative two-dimensional topology of the human herpesvirus-8 GPCR;

Fig. 8 is a diagram of the putative two-dimensional topology of the chimera-ThrR/HHV8 GPCR as it is predicted to be in the cell surface membrane of transfected COS-1 cells; and

Fig. 9 is a plasmid map of pcDNA3PROLACFLAGhTHRR/hFSHR.

#### DETAILED DESCRIPTION

The invention provides a strategy that is designed to discover small peptides that will activate any G protein-coupled receptor (GPCR) or inactivate any constitutively active GPCR. A constitutively active



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receptor is one that signals in the absence of an agonist. These peptides could serve as lead chemicals for design of clinically useful drugs or could be used to identify the natural ligand or physiologic function of "orphan" receptors, that is, putative receptors that have been identified (cloned) but for which the function is unknown. The discovery of peptides that could serve these functions may be accomplished with an approach that uses combinatorial peptide libraries. With this approach, millions of random peptides of a given length are tested for activity in the context of a library and those that activate GPCRs or inactivate constitutively active GPCRs are discovered. As stated above, this approach may have enormous potential for clinical applications because a number of peptide agonists of GPCRs are currently used therapeutically and diagnostically. In the shorter term, however, this technology will yield reagents that could be used by pharmaceutical companies to identify ligands for or functions of "orphan" receptors.

To discover small peptides that can serve as agonists for GPCRs, a combinatorial peptide library is constructed that expresses random pentapeptides tethered to the seven TM helical bundle of any GPCR. A pentapeptide library was chosen based on the fact that TRH is a tripeptide that is blocked at both ends (3+2 (for block) =5) and the resulting number of clones is workable.

The library contains all 20 natural amino acids at each of the five positions and therefore has a complexity of  $20^5 = 3.2 \times 10^6$  possible combinations. To this end the complementary DNA (cDNA) sequence that normally encodes any GPCR's N-terminal EC domain is substituted by a DNA sequence that encodes the N-terminal ectodomain of a self-activating receptor such as the thrombin receptor. Thrombin receptor (ThrR) is a GPCR that is activated by a mechanism that is

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different from most GPCRs. Thrombin is a serine protease that binds to and cleaves its receptor's N-terminal end at a specific site, exposing a new N-terminus that acts as a peptide agonist tethered to the remainder of the receptor molecule. The chimeric ThrR/GPCR has the variable pentapeptide sequence substituting for the native peptide sequence that is normally unmasked by thrombin action and constitutes the ThrR peptide agonist, but retains thrombin binding sequences and the thrombin-specific cleavage site. Therefore, the N-terminus of expressed receptors is cleaved by thrombin at the appropriate location exposing a new N-terminus that is made of the variable pentapeptide segment of the library tethered to the remainder of the GPCR. As used herein, a receptor that operates in this manner is referred to as a self-activating receptor since a ligand of the receptor cleaves the receptor to expose a natural peptide agonist which activates the receptor. Thrombin is the most well known of such self-activating receptors, but the invention can be readily practiced using other such receptors (e.g., the protease activated receptor or a synthetic receptor).

The cDNA sequence encoding the new N-terminus of the chimeric ThrR/GPCR, consisting of a prolactin leader or signal peptide, followed by the FLAG epitope, followed by the N-terminus of the mature human ThrR, where the pentapeptide library is constructed, is constructed by gene synthesis. The cDNA sequence consists of a DNA segment of approximately 300 base pairs encoding 100 amino acids that is ligated in frame through an appropriate restriction endonuclease cleavage site created by polymerase chain reaction (PCR) in the cDNA of any GPCR at a position encoding the amino acids that constitute the transition between the N-terminus and the first TM domain. After ligation into a mammalian expression vector, *Escherichia coli* is

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transformed by electroporation and the transformants are subdivided into pools whose maximal workable complexity is determined according to the efficiency of mammalian cell transfection and/or sensitivity of the detection system.

5           Amplified reporter systems based on the second messenger systems triggered by the GPCR are used. For discovery of agonists, the assay is based on gene induction in COS-1 cells using  $\beta$ -galactosidase as  
10   a reporter gene in a single cell assay. This assay takes advantage of the amplification of the enzyme activity of the reporter, with an easily determined color reaction as endpoint, and of the expression of a single receptor clone with its tethered agonist in COS-  
15   1 cells because of replication of the plasmids introduced. The signal is increased because the construct used has a nuclear localization signal ligated to the  $\beta$ -galactosidase that allows the protein to concentrate in the nucleus (Hersh et al. 1995).  
20   Single clones that exhibit activation of chimeric ThrR/GPCR after thrombin addition to cleave the N-terminus and expose the tethered agonist, as measured by increased color reaction, are isolated using sib  
25   selection, which consists of successive subdivision and amplification of positive pools of clones. A number of other reporter systems can also be used. These include, but are not limited to, analysis of acute effects of agonist using *Xenopus laevis* oocytes in which one measures changes in membrane conductance -  
30   using calcium-activated chloride conductance for phosphoinositide (PI) cascade or cAMP-activated chloride conductance through cystic fibrosis transmembrane regulator (CFTR) that is co-expressed for cAMP cascade; induction of genes in COS-1 cells that  
35   yield protein products that are displayed in the cytoplasm or on the surfaces of cells and visualized by immunofluorescence (by microscopy or fluorescence

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activated cell sorting) or immunocytochemistry; and analysis of acute effects on elevation of cytoplasmic calcium using fluorescence indicators.

5 To discover small peptides that can serve as agonists or small peptides that can serve as negative antagonists (or inverse agonists) for GPCRs, a second type of combinatorial peptide library is constructed that expresses random pentapeptides tethered to the seven TM helical bundle of a given GPCR that is  
10 different from the one described above to discover agonists but is based on the same principles. This library also contains all 20 natural amino acids at each of the five positions and therefore has a complexity of  $20^5 = 3.2 \times 10^6$  possible combinations. In  
15 this library, however, the cDNA sequence that normally encodes GPCR's N-terminal EC domain is substituted by a DNA sequence that encodes the self-activating receptor's (e.g., thrombin's) N-terminal ectodomain but without the domain that usually is cleaved to reveal  
20 the tethered peptide. In this library, the chimeric ThrR/GPCR has the variable pentapeptide sequence substituting for the native peptide sequence that is normally unmasked by thrombin action exposed as the N-terminus of all receptors. Therefore, the N-terminus  
25 of expressed receptors is a random pentapeptide that can act as an agonist of a GPCR or as a negative antagonist with regard to the constitutive activity of some GPCRs. With regard to the negative antagonists, in contrast to looking for stimulation of a GPCR  
30 signalling response, monitoring is for inactivation of a "basal" activity.

A two-reporter system is used for discovery of negative antagonists. The second reporter gene is used to identify cells that have been transfected and  
35 are expressing foreign proteins and to distinguish them from cells that have not been transfected and are not expressing foreign proteins. This is a crucial

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distinction for this approach because differentiation between cells that have the capacity to express the specific reporter gene but are not because transcription has been inhibited and cells that are not expressing the reporter gene because they are not transfected is necessary. The same reporter genes for GPCR-specific effects as for the discovery of agonist peptides are used. The nonspecific reporter for transfection is a construct containing a mutant of the human placental alkaline phosphatase gene (Tate et al. 1990) that is targeted to the cytoplasm under the control of a cytomegalovirus promoter. Thus, one can monitor for 3 types of cells: 1) cells in which  $\beta$ -galactosidase is expressed at high levels in the nucleus and alkaline phosphatase is expressed in the cytoplasm - these are transfected cells that do not express receptors that contain a peptide that has negative antagonistic activity because expression of  $\beta$ -galactosidase is induced by the constitutive signalling activity of the GPCR; 2) cells in which  $\beta$ -galactosidase is not expressed in the nucleus and alkaline phosphatase is not expressed in the cytoplasm - these are cells that have not been transfected; and 3) cells in which  $\beta$ -galactosidase is not expressed or is expressed at low levels in the nucleus and alkaline phosphatase is expressed in the cytoplasm - these are transfected cells that express receptors that contain a peptide that has negative antagonistic activity. The approach to sib selection is identical to that outlined above.

A yeast (*Saccharomyces cerevisiae*) bioassay system that is responsive to activation of GPCRs or to inactivation of constitutively active GPCRs can also be used to screen the tethered, combinatorial peptide library. This bioassay is based on the finding that mammalian GPCRs expressed in yeast will regulate the endogenous signal transduction cascades (Dohlman et al.

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1991), in particular the pathway for regulation of proliferation (King et al. 1990). A sensitive and specific yeast expression system permits powerful genetic selection methods, which use modifications in the endogenous pheromone response pathways (Price et al. 1995; Price et al. 1996), to be developed for use with the screening methods of the subject invention. The pheromone signalling cascade in yeast uses one of two GPCRs {for (STE2) or a mating factor (STE3)} to couple to a heterotrimeric G protein, which is comprised of (GPA1), (STE4) and (STE18) subunits, to activate a protein kinase signalling cascade that leads to cell cycle arrest, which is mediated by FAR1, and activation of pheromone-responsive genes, such as FUS1. SST2 is another important member of this signalling pathway because it serves to desensitize (or "turn off") the pathway. Several members of this pathway can be modified to improve the sensitivity and assay of GPCRs. This system provides markedly greater ease of assay and permits the screening of hundreds of thousands of recombinant GPCR clones simultaneously. Systems can be developed that can be used to screen for agonist and negative antagonist probes/drugs. The major advantage of this type of assay system over those usually employed to screen numerous potential probes/drugs rapidly, which is necessary for the application of the method of the subject invention, is that it relies on a response in a single yeast cell and will identify the responsive cell in a population of millions of cells.

One assay will be a minor modification of the previously published yeast expression system to assay for activation of GPCRs in which FAR1 and SST2 genes were inactivated and a FUS1-HIS3 gene is used for selection of cells expressing activated GPCRs on a medium deficient in histidine (Price et al. 1995). The changes will involve only adapting the system so that

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it will allow high efficiency transformation of yeast cells with a library that contains 3.2 million different GPCRs. The second assay will be modified more extensively so that it will measure constitutively activated GPCRs that are inactivated. One approach to this type of assay will involve using yeast cells in which the FAR1 gene is intact so that constitutively active GPCRs will cause cells to be arrested in the cell cycle. Cells in which the GPCR has been inactivated will not exhibit growth arrest but will proliferate as normal haploid cells in the absence of mating factor.

The invention thus provides a method of identifying peptide agonists or negative antagonists of a G protein coupled receptor of interest. The method comprises expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell, and monitoring the cell to determine whether the peptide is an agonist or negative antagonist of the G protein coupled receptor of interest.

In one embodiment for identifying peptide agonists, the expression of a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises preparing a G protein coupled receptor construct, introducing the G protein coupled receptor construct into a cell, allowing the cell to express the G protein coupled receptor encoded thereby, and exposing the cell to a ligand of a self-activating receptor, wherein the ligand cleaves the G protein coupled receptor construct so as to expose the inserted peptide of the peptide library. The G protein coupled receptor construct for identifying a peptide agonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached

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to the nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which is a peptide agonist for activating the self-activating receptor; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

One embodiment of a G protein coupled receptor construct for identifying a peptide agonist of the G protein coupled receptor is shown in Fig. 4. Referring to Figs. 1-4, the construct involves three parts based on a nucleic acid molecule encoding a G protein coupled receptor (10) (Fig. 1), a nucleic acid molecule encoding a thrombin receptor (12) (Fig. 2), and a nucleic acid molecule encoding a peptide (14) of a peptide library (Fig. 3). Referring to Fig. 1, the G protein coupled receptor (10) includes an amino terminus (16). Referring to Fig. 2, the thrombin receptor (12) also includes an amino terminus (18). Within the amino terminus (18) of the thrombin receptor (12) is a portion (20) which is a peptide agonist for the thrombin receptor. When the thrombin receptor is exposed to thrombin, thrombin cleaves the amino terminal part of the molecule (22) leaving the portion (20) which is a peptide agonist exposed. The portion (20) reacts with the remainder of the thrombin molecule and binds thereto, activating the thrombin receptor. Referring to Fig. 3, the peptide (14) of a peptide library is shown.

Fig. 4 shows one embodiment of the G protein coupled receptor construct for identifying a peptide agonist according to the subject invention positioned within a cellular membrane (24). The construct includes a nucleic acid molecule encoding the G protein coupled receptor (10) but a portion of the nucleic acid molecule which encodes the amino terminus of the



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receptor is deleted. In its place, the amino terminus (18) of the thrombin receptor is inserted. Within the amino terminus (18) of the thrombin receptor, the portion which is a peptide agonist has been deleted and replaced with the peptide (14) of the peptide library. Thus, the G protein coupled receptor construct has the backbone of a selected G protein coupled receptor, with an amino terminus of the thrombin receptor. However, the normal peptide agonist of the thrombin receptor has been replaced by a peptide library. Thus, when the G protein coupled receptor construct of the subject invention is exposed to thrombin, thrombin will cleave the amino terminal part (22) of the construct leaving the peptide (14) of the peptide library exposed. If the exposed peptide is an agonist of the G protein coupled receptor, the receptor will be turned on.

In a further embodiment for identifying a peptide negative antagonist, the G protein coupled receptor of interest is a constitutively active G protein coupled receptor and the expression of a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises preparing a constitutively active G protein coupled receptor construct, introducing the constitutively active G protein coupled receptor construct into a cell, and allowing the cell to express the constitutively active G protein coupled receptor encoded thereby. The constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a constitutively active G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the constitutively active G protein coupled receptor at the deleted first amino terminus,

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the second amino terminus having a deleted portion which includes a peptide agonist for activating the self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and  
5 a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

The constitutively active G protein coupled receptor construct for identifying a peptide negative  
10 antagonist of the constitutively active G protein coupled receptor is shown in Fig. 5, positioned within a cellular membrane (24). The construct includes a nucleic acid molecule encoding the G protein coupled receptor (10) but a portion of the nucleic acid  
15 molecule which encodes the amino terminus of the receptor is deleted. In its place, the amino terminus (18) of the thrombin receptor is inserted. Within the amino terminus (18) of the thrombin receptor, the portion which is a peptide agonist has been deleted as  
20 well as any amino acids positioned amino terminally to the peptide agonist which are normally cleaved by thrombin, and replaced with the peptide (14) of the peptide library. Thus, the constitutively active G protein coupled receptor construct has the backbone of  
25 a selected G protein coupled receptor, with an amino terminus of the thrombin receptor. However, the normal peptide agonist of the thrombin receptor has been replaced by a peptide library and the peptide is always exposed. If the exposed peptide is a negative  
30 antagonist of the constitutively active G protein coupled receptor, the receptor will be turned off by the exposed peptide.

In a still further embodiment for identifying peptide agonists, the expression of a peptide of a  
35 peptide library tethered to a G protein coupled receptor of interest in a cell comprises preparing a G protein coupled receptor construct, introducing the G

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protein coupled receptor construct into a cell, and allowing the cell to express the G protein coupled receptor encoded thereby. The G protein coupled receptor construct for identifying a peptide agonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which includes a peptide agonist for activating the self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion. This G protein coupled receptor construct for identifying a peptide agonist of a G protein coupled receptor has the same structure as the construct shown in Fig. 5 except that the G protein coupled receptor (10) is not a constitutively active receptor.

The Examples which follow relate to particular GPCRs, such as the human calcitonin receptor, the human follicle-stimulating hormone receptor, and a GPCR of human herpesvirus-8. However, as should be readily apparent to those of ordinary skill in the art, this invention is equally applicable to any GPCR. GPCRs are the largest family of cell surface receptors and act indirectly to regulate the activity of a separate plasma membrane-bound target protein, which can be an enzyme or an ion channel. The interaction between the receptor and the target protein is mediated by a third protein, called a trimeric GTP-binding regulatory protein (G protein). The activation of the target protein either alters the conformation of

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one or more intracellular mediators (if the target protein is an enzyme) or alters the ion permeability of the plasma membrane (if the target protein is an ion channel).

5 GPCRs include, for example, the alpha-adrenergic receptors, the beta-adrenergic receptors, dopaminergic receptors, serotonergic receptors, muscarinic cholinergic receptors, peptidergic receptors, and the thyrotropin releasing hormone  
10 receptor. GPCRs are characterized by a seven transmembrane-spanning topology (see Figs. 1, 2, 4-8). As used herein, the amino terminus of a GPCR refers to that portion of the GPCR which is extracellular, extending from the amino end of the GPCR to the first  
15 transmembrane domain (the amino terminus is depicted in Figs. 4 and 5).

The various G protein coupled receptor constructs of the subject invention include the amino terminus of a self-activating receptor as defined  
20 herein. In one embodiment, the self-activating receptor is the thrombin receptor. The amino acid sequence of this amino terminus of the thrombin receptor is shown in SEQ ID NO:1, with amino acid residues 9 to 13 of SEQ ID NO:1 representing the  
25 natural peptide agonist of the thrombin receptor. These residues (9 to 13 of SEQ ID NO:1) are replaced with the peptide library in accordance with the subject invention. In one embodiment of the G protein coupled receptor construct, the amino acids normally cleaved by  
30 thrombin (residues 1 to 8 of SEQ ID NO:1) are also replaced by the peptide of the peptide library.

SEQ ID NO:1:

LDATLDPRSFLLRNPNDKYEPFWEDEEKNESGLTEYRLVSINKSSPLQK  
35 CIPAFISEDASGYL

In one embodiment discussed in the Examples, the G protein coupled receptor construct is of a human

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calcitonin receptor (see Fig. 6). The human calcitonin receptor construct according to the subject invention has an amino acid sequence as shown in SEQ ID NO:44, wherein amino acid residues 47 to 51 of SEQ ID NO:44 are the peptide of a peptide library, amino acid residues 1 to 101 of SEQ ID NO:44 are the second amino terminus, and amino acid residues 102 to 429 of SEQ ID NO:44 are the nucleic acid molecule encoding the human calcitonin receptor with the first amino terminus deleted.

SEQ ID NO:44:

MDSKGSSQKGSRLLLLLLVSNLLLCQGVVSDYKDDDDKLDATLDPXXXXXNPNDKYEPF  
WEDEFKNESGLTEYRLVSINKSSPLQKQLPAFISEDASGYLVLYLAIVGHSLSIFTLVI  
SLGIFVFFRSLGCQRVTLHKNMFLTYILNSMIIIIHLVEVVPNGELVRRDPVSKILHFF  
HQYMMACNYFWMLCEGIYLHTLIVVAVFTEKQRLRWYLLGWGFPLVPTTIHAITRAVYF  
NDNCWLSVETHLLYIIHGPMVMAALVNVFFLLNIVRVLVTKMRETHEAESHYLKAVKAT  
MILVPLLGIQFVVFWRPSNKMGLGIYDYVMHSLIHFGGFFVATYICFCNNEVQTTVKRQ  
WAQPKIQWNQWRGRRPSNRSARAAAAAAEAGDIPYIYCHQELRNEPANNOGEESAEIPL  
NIIEQESSA

In a further embodiment discussed in the Examples, the G protein coupled receptor construct is of a human follicle stimulating hormone receptor. The human follicle stimulating hormone receptor construct has an amino acid sequence as shown in SEQ ID NO:2, wherein amino acid residues 47 to 51 of SEQ ID NO:2 are the peptide of a peptide library, amino acid residues 39 to 101 of SEQ ID NO:2 are the second amino terminus, and amino acid residues 102 to 436 of SEQ ID NO:2 are the nucleic acid molecule encoding the human follicle stimulating hormone receptor with the first amino terminus deleted.

SEQ ID NO:2:

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MDSKGSSQKGSRLLLLLLVSNLLLCQGVVSDYKDDDDKLDATLDPXXXXXNPNDKYEPFWEDEBK  
 NESGLTEYRLVSINKSSPLQKQLPAFISEDASGYLGYNILRVLIWFISILAITGNIIVLVILTTSQ  
 YKLTVPFRFLMCNLAFLADLCIGIYLLLIASVDIHTKSQYHNYAIDWQTGAGCDAAGFFTVFASLSV  
 YTLTAITLERWHTITHAMQLDCKVQLRHAASVMVMGWIFAFAAALFPIFGISSYMKVSIICLPMDID  
 5 SPLSQLYVMSLLVLNVLAFFVICGCIYIHYLTVRNPNIVCSSSDTRIAKRMAMLIPTDFLCMAPIS  
 FFAISASLKVPLITVSKAKILLVLFHPINSCANPFLYAIFTKNFRDFFILLSKCGCYEMQAQIYR  
 TETSSTVHNTHPRNGHCSSAPRVTVNGSTYILVPLSHLAQN

As used herein, the term "as shown in" when  
 10 used in conjunction with a SEQ ID NO for a nucleotide  
 sequence refer to a nucleotide sequence which is  
 substantially the same nucleotide sequence, or  
 derivatives thereof (such as deletion and hybrid  
 variants thereof, splice variants thereof, etc.).  
 15 Nucleotide additions, deletions, and/or substitutions,  
 such as those which do not affect the translation of  
 the DNA molecule, are within the scope of a nucleotide  
 sequence as shown in a particular nucleotide sequence  
 (i.e. the amino acid sequence encoded thereby remains  
 20 the same). Such additions, deletions, and/or  
 substitutions can be, for example, the result of point  
 mutations made according to methods known to those  
 skilled in the art. It is also possible to substitute  
 a nucleotide which alters the amino acid sequence  
 25 encoded thereby, where the amino acid substituted is a  
 conservative substitution or where amino acid homology  
 is conserved. It is also possible to have minor  
 nucleotide additions, deletions, and/or substitutions  
 which do not alter the function of the resulting GPCR.  
 30 These are also within the scope of a nucleotide  
 sequence as shown a particular nucleotide sequence.

Similarly, the term "as shown in" when used  
 in conjunction with a SEQ ID NO for an amino acid  
 sequence refers to an amino acid sequence which is  
 35 substantially the same amino acid sequence or  
 derivatives thereof. Amino acid additions, deletions,  
 and/or substitutions which do not negate the ability of  
 the resulting protein (or peptide) to form a functional

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protein (or peptide) are within the scope of an amino acid sequence as shown in a particular amino acid sequence. Such additions, deletions, and/or substitutions can be, for example, the result of point mutations in the DNA encoding the amino acid sequence, such point mutations made according to methods known to those skilled in the art. Substitutions may be conservative substitutions of amino acids. Two amino acid residues are conservative substitutions of one another, for example, where the two residues are of the same type. In this regard, alanine, valine, leucine, isoleucine, glycine, cysteine, phenylalanine, tryptophan, methionine, and proline, all of which are nonpolar residues, are of the same type. Serine, threonine, tyrosine, asparagine, and glutamine, all of which are uncharged polar residues, are of the same type. Another type of residue is the positively charged (basic) polar amino acid residue, which includes histidine, lysine, and arginine. Aspartic acid and glutamic acid, both of which are negatively charged (acidic) polar amino acid residues, form yet another type of residue. Further descriptions of the concept of conservative substitutions are given by French and Robson 1983, Taylor 1986, and Bordo and Argos 1991.

As further used herein, the term "as shown in" when used in conjunction with a SEQ ID NO for a nucleotide or amino acid sequence is intended to cover linear or cyclic versions of the recited sequence (cyclic referring to entirely cyclic versions or versions in which only a portion of the molecule is cyclic, including, for example, a single amino acid cyclic upon itself), and is intended to cover derivative or modified nucleotide or amino acids within the recited sequence. For example, those skilled in the art will readily understand that an adenine nucleotide could be replaced with a methyladenine, or a

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cytosine nucleotide could be replaced with a methylcytosine, if a methyl side chain is desirable. Nucleotide sequences having a given SEQ ID NO are intended to encompass nucleotide sequences containing these and like derivative or modified nucleotides, as well as cyclic variations. As a further example, those skilled in the art will readily understand that an asparagine residue could be replaced with an ethylasparagine if an ethyl side chain is desired, a lysine residue could be replaced with a hydroxylysine if an OH side chain is desired, or a valine residue could be replaced with a methylvaline if a methyl side chain is desired. Amino acid sequences having a given SEQ ID NO are intended to encompass amino acid sequences containing these and like derivative or modified amino acids, as well as cyclic variations. Cyclic, as used herein, also refers to cyclic versions of the derivative or modified nucleotides and amino acids.

As further used herein, a nucleic acid molecule can be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), the latter including messenger RNA (mRNA). The nucleic acid can be genomic or recombinant, biologically isolated or synthetic.

The DNA molecule can be a cDNA molecule, which is a DNA copy of an mRNA encoding the protein.

The G protein coupled receptor construct of the subject invention can be expressed in suitable host cells using conventional techniques. Any suitable host and/or vector system can be used to express the GPCR construct. For in vitro expression, bacterial hosts (for example, *Escherichia coli*) and mammalian hosts (for example, COS cells) are preferred. For screening using the GPCR construct in which the inserted peptide is always exposed, yeast cells are preferred. The use of yeast cells as a host for expression of the GPCR construct allows for the



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screening for negative antagonists of constitutively active GPCRs or for the screening of agonists of GPCRs. Expression of the construct is desirable to identify peptide agonists and negative antagonists of the GPCR, which can then be used for study and/or research purposes, as well as for therapy of inherited or acquired human disorders related to GPCR function.

Techniques for introducing the construct into the host cells may involve the use of expression vectors which comprise the nucleic acid molecule encoding the construct. These expression vectors (such as plasmids and viruses) can then be used to introduce the nucleic acid molecule into suitable host cells. For example, DNA encoding the construct can be injected into the nucleus of a host cell or transformed into the host cell using a suitable vector, or mRNA encoding the construct can be injected directly into the host cell, in order to obtain expression of the GPCR construct in the host cell.

Various methods are known in the art for introducing nucleic acid molecules into host cells. One method is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles (or RNA is injected directly into the cytoplasm of cells). Alternatively, DNA can be incubated with an inert carbohydrate polymer (e.g. dextran) to which a positively charged chemical group (e.g. diethylaminoethyl ("DEAE")) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles, in turn, stick to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell. In another method, cells efficiently take in DNA in the form of a precipitate with calcium

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phosphate. In electroporation, cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures (passage through these vesicles may sometimes destroy or damage DNA). DNA can also be incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, used primarily with plant cells and tissues, DNA is absorbed to the surface of tungsten microprojectiles and fired into cells with a device resembling a shotgun.

Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. Since viral growth depends on the ability to get the viral genome into cells, viruses have devised clever and efficient methods for doing it. Various viral vectors have been used to transform mammalian cells, such as vaccinia virus, adenovirus, and retrovirus.

As indicated, some of these methods of transforming a cell require the use of an intermediate plasmid vector. U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

Host cells into which the nucleic acid encoding the construct has been introduced can be used

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to produce (i.e. to functionally express) the GPCR construct. The cell can then be monitored to determine whether the peptide tethered to the GPCR is an agonist or negative antagonist (in the case of a constitutively active GPCR) of the GPCR. The method of monitoring can be chosen based on the signalling pathway of the GPCR, or the construct can further include marker or reporter systems as discussed in further detail below. For example, if the G protein coupled receptor signals through an ion channel pathway, the monitoring can comprise detecting levels of the ion within the cell. If the G protein coupled receptor signals through a calcium ion channel pathway, the cell to be used can be a *Xenopus* oocyte and the monitoring can comprise voltage clamp analysis. If the G protein coupled receptor signals through a cyclic adenosine monophosphate pathway, the monitoring can comprise detecting levels of cyclic adenosine monophosphate within the cell.

The invention further provides a cell comprising the G protein coupled receptor construct of the subject invention, as well as an expression vector comprising the construct. A host cell comprising the expression vector is also provided. Such expression vectors include a plasmid and a virus. Preferably, the cell into which the construct or expression vector (comprising the construct) is introduced is a *Xenopus* oocyte, a mammalian cell (such as COS-1 cells; see Gershengorn and Osman 1996), or a yeast cell.

30

#### EXAMPLE I

##### Peptide Agonists of hFSH-R

A combinatorial peptide library was constructed that expresses random pentapeptides tethered to the seven transmembrane helical bundle of the human follicle-stimulating hormone receptor

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(hFSH-R). This library encompasses all 20 natural amino acids at each of the five positions, and, therefore, has a complexity of  $20^5 = 3.2 \times 10^6$  possible combinations. To this end, the complementary DNA sequence that normally encodes the hFSH-R's amino terminal extracellular domain was substituted by a DNA sequence that encodes the thrombin receptor's amino terminal ectodomain. The chimeric human THR-R/FSH-R has the variable pentapeptide sequence substituting for the native peptide sequence that is normally unmasked by thrombin action and constitutes the thrombin receptor agonist peptide, but it retains thrombin binding sequences and the thrombin specific cleavage site. Therefore, the amino terminus of expressed receptors is cleavable by thrombin at the appropriate location exposing a new amino terminus that is made of the variable pentapeptide segment of the library tethered to the transmembrane domains of hFSH-R.

To monitor for cell surface expression and efficient cleavage by thrombin of the amino terminal end of the chimeric receptors, an epitope-tag to which antibodies are available was positioned proximally to the thrombin cleavage site. Antibodies that recognize thrombin receptor amino terminus distal to the position corresponding to the library are also available. Consequently, chimeric receptors expressed on the cell surface are detectable by the appropriate use of both types of specific antibodies before thrombin treatment, but only with antibodies against the distal part after thrombin treatment.

The amino acid sequence of the chimeric human THR-R/FSH-R is shown in SEQ ID NO:2:

MDSKGSSQKGSRLLLLLVVSNNLLCQGVVSDYKDDDDKLDATLDPRXXXXXNPNDKYEPFWEDEEK  
NESELT EYRLVSINKSSPLQKQLPAFISEDASGYLGYNILRVLIWFISILAITGNIIVLVILETSQ  
YKLT VPRFLMCNLAFADLCIGIYLLLIASVDIHTKSQYHNYAIDWQTGAGCDAAGFFT VFASELSV  
YTLTAITLERWHTITHAMQLDCKVQLRHAASVMVMGWIFAFAAALFP IFGISSYMKVSICLPMDID  
SPLSQLVMSLLVLNVLAFFVICGCIYIHYLTVRNPNI VSSSDTRI AKRMAMLI FTDFLCMAPIS

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FFAISASLKVPLITVSKAKILLVLFHPINSCANPFLYAIFTKNFRRDFFILLSKCGCYEMQAQIYR  
TETSSTVHNTHPRNGHCSSAPRVINGSTYILVPLSHLAQN

The construct consists of 436 amino acids: amino acid  
5 residues 1-30 represent the prolactin signal peptide  
(SEQ ID NO:6:MDSKGSSQKGSRLLLLLLVSNLLLCQGVVS); residues  
31-38 represent the FLAG epitope (SEQ ID  
NO:4:DYKDDDDK); residues 39-101 represent amino acids  
from the hTHR receptor of which residues 47-51  
10 represent the pentapeptide (SEQ ID NO:5:XXXXX) and  
residues 57-74 represent the hirudin epitope; and  
residues 102-436 represent amino acids from the hFSH  
receptor of which residues 108-128 represent  
transmembrane domain 1, residues 140-162 represent  
15 transmembrane domain 2, residues 185-206 represent  
transmembrane domain 3, residues 227-250 represent  
transmembrane domain 4, residues 270-291 represent  
transmembrane domain 5, residues 316-338 represent  
transmembrane domain 6, and residues 350-371 represent  
20 transmembrane domain 7. The signal peptide cleavage  
site lies between amino acid residues 30 and 31 of SEQ  
ID NO:2, and the thrombin cleavage site lies between  
amino acid residues 46 and 47 of SEQ ID NO:2. Cleavage  
with thrombin thus exposes the pentapeptide that is  
25 amino acid residues 47-51 of SEQ ID NO:2.

The construction of the DNA sequence encoding  
the amino acid sequence shown in SEQ ID NO:2 took  
several steps that are described below:

- 1) Construction of a sequence encoding the  
30 prolactin signal peptide (SEQ ID NO:3) followed by a  
FLAG epitope-tag (SEQ ID NO:4: DYKDDDDK) placed  
immediately upstream of the putative mature sequence  
for human thrombin receptor amino terminus ectodomain  
(from amino acids 34 to 95, SEQ ID NO:1) was produced  
35 by gene synthesis using standard techniques as  
described (Nussenzveig 1994). Synthetic  
oligonucleotides obtained for the prolactin leader  
sequence-FLAG epitope-tag construction have the

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following sequences: coding strand PROLAC-1: SEQ ID NO:6: 5'- AAT TCC ACC ATG GAC TCC AAG GGC TCG AGC CAG AAG GGA TCT AGA CTG CT -3' ; complementary strand PROLAC-2: SEQ ID NO:7: 5'- PO<sub>4</sub>- CAG CAG CAG TCT AGA TCC CTT CTG GCT CGA GCC CTT GGA GTC CAT GGT GG -3' ; coding strand PROLAC-3: SEQ ID NO:8: 5'- PO<sub>4</sub>- G CTG CTG CTG GTG GTG AGC AAC CTG CTG CTG TGC CAG GGC GTC GTG -3' ; complementary strand PROLAC-4: SEQ ID NO:9: 5'- PO<sub>4</sub>- CGC TCA CGA CGC CCT GGC ACA GCA GCA GGT TGC TCA CCA CCA GCA G -- 3' ; FLAG-SENSE: SEQ ID NO:10: 5'- PO<sub>4</sub>- AGC GAC TAC AAG GAC GAC GAC GAC AAG CTT CCT GCC TTT T -3' ; FLAG-ANTI-SENSE: SEQ ID NO:11: 5'- CGA AAA GGC AGG AAG CTT GTC GTC GTC GTC CTT GTA GT -3'. The pair of oligonucleotides PROLAC-1/PROLAC-2; PROLAC-3/PROLAC-4; and FLAG-SENSE/FLAG-ANTI-SENSE were annealed separately at 20  $\mu$ M final oligonucleotide concentration, by heating at 95  $^{\circ}$ C for 5 min and cooling to 4  $^{\circ}$ C at a rate of 1  $^{\circ}$ C every 3 min, in 20 mM Tris-Cl pH 7.6 and 10 mM MgCl<sub>2</sub> buffer, using a thermal controller apparatus.

Double stranded DNA was purified by agarose gel electrophoresis using the Mermaid™ purification system (Bio 101). Purified double stranded oligonucleotides were ligated using equal molar concentrations. Ligation products were digested with HindIII after heat inactivation of T4 DNA ligase. The resulting 125 bp larger fragment was purified by agarose gel electrophoresis using the Mermaid™ kit. Fragment of interest was subcloned into EcoRI and HindIII sites of pBSSKII(+). Correctness of the sequence was verified by dideoxynucleotide sequencing method using Circumvent sequencing kit (New England Biolabs, Inc.).

2) Construction of a sequence encoding the human thrombin receptor amino terminus from amino acid residue F<sup>55</sup> to L<sup>96</sup> (residues 60-101 of SEQ ID NO:2) was obtained by assembling four synthetic overlapping oligonucleotides containing gaps from 10 to 33 nucleotides: coding strand THRR-1: SEQ ID NO:12: 5' -

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TAT GCC ACC TTT TGG GAG GAT GAG GAG AAA AAT GAA AGT GGG  
TTA ACT GAA TAC - 3'; complementary strand THRR-2: SEQ  
ID NO:13: 5' - TG AAG AGG ACT GCT TTT ATT GAT GGA GAC  
TAA TCT GTA TTC AGT TAA CCC ACT TTC - 3'; coding strand  
5 THRR-3: SEQ ID NO:14: 5' - C AAT AAA AGC AGT CCT CTT  
CAA AAA CAA CTT CCT GCA TTC ATC TCA GAA GAT GCC - 3';  
complementary strand THRR BstEII : SEQ ID NO:15: 5' -  
GT CAG GTA ACC GGA GGC ATC TTC TGA GAT GAA TGC AAG -  
3'. Oligonucleotide THRR BstEII inadvertently mutated  
10 hTHRR codon for P<sup>85</sup> into L. Oligonucleotides THRR-2 and  
THRR-3 were phosphorylated enzymatically using T4  
polynucleotide kinase in 50 mM Tris-HCl pH 7.5, 10 mM  
MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mM ATP and 25 µg/ml BSA.  
Oligonucleotides THRR-1, THRR-2, THRR-3 and THRR BstEII  
15 were annealed at a final concentration of 10 µM in 20  
mM Tris-Cl pH 7.6, 10 mM MgCl<sub>2</sub> buffer by heating at 95  
°C for 5 min and cooling to 4 °C at a rate of 1 °C per 8  
min using a thermal controller apparatus. The gaps  
between annealed oligonucleotides were filled-in using  
20 T4 DNA polymerase. Reaction was performed at a final  
concentration of 2.5 µM oligonucleotides, 400 µM dNTPs,  
50 mM NaCl, 15 mM Tris-HCl, 12.5 mM MgCl<sub>2</sub>, 1 mM  
dithiothreitol, 50 µg/ml BSA, pH 7.9 at 25 °C for 60  
min. Reaction was stopped with 25 mM EDTA and enzyme  
25 was heat inactivated at 65 °C for 60 min. T4 DNA  
polymerase was selected not only to avoid strand  
displacement of the overlapping oligonucleotides but  
also because of its 3' to 5' exonuclease activity to  
correct the inadvertent mutation P<sup>85</sup> to L.  
30 3) Construction of a nucleotide sequence  
encoding amino acid residues from G<sup>361</sup> to N<sup>694</sup> (residues  
102-436 of SEQ ID NO:2) followed by the stop codon of  
the hFSHR was obtained by standard taq polymerase PCR  
method using the following pair of oligonucleotides: i)  
35 coding strand FSHR BstEII: SEQ ID NO:16: 5' - T GAA GGT  
TAC CTG GGG TAC AAC ATC CTC AGA GTC C - 3'; and ii)  
complementary strand NotI 2170: SEQ ID NO:17: 5' - TCA

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CGC GGC CGC TTA GTT TTG GGC TAA ATG ACT TAG AGG - 3'.  
The resultant PCR product creates *Bst*EII and *Not*I sites  
at the 5' and 3' ends of the coding strand,  
respectively. The *Bst*EII site was used to connect the  
5 hFSHR sequence in frame with the amino terminus  
ectodomain of the hTHRR. The *Not*I site was used to  
connect the chimeric construct to the expression  
vector. Resulting PCR product was cloned into a  
pBSSKII(+)*AT* vector prepared to receive PCR fragments  
10 containing non-template dependent addition of 3'  
A-overhangs. pBSSKII(+)*AT* vector, that contains 3'  
T-overhangs, was obtained by ligating phosphorylated  
oligonucleotides *AT*-SENSE: SEQ ID NO:18: 5' - PO<sub>4</sub> AAT  
TCG GCT T - 3' and *AT*-ANTI-SENSE: SEQ ID NO:19: 5' - PO<sub>4</sub>  
15 AGC CG - 3' into pBSSKII(+)*AT* vector cut with *Eco*RI. A  
clone was selected with the orientation that places the  
newly created *Bst*EII site closer to the *Sac*I site of  
the vector and the *Not*I site of the insert closer to  
the *Kpn*I site of the vector.  
20 4) Modification of the hFSHR construct obtained  
in item # 3 through the production of silent mutations  
to destroy the two *Pfl*MI sites originally present at  
positions 1,379 and 2,080 of the hFSHR cDNA. hFSHR DNA  
sequence was modified by PCR mutagenesis, using the  
25 construct obtained in item # 3 as a template in a  
standard PCR reaction with *taq* polymerase and the  
following three pairs of primers: i) *Pvu*I 1379 -  
ANTI-SENSE: SEQ ID NO:20: 5' - CA GTC GAT CGC ATA GTT  
GTG ATA TTG GCT C - 3' and vector REVERSE PRIMER: SEQ  
30 ID NO:21: 5' - AAC AGC TAT GAC CAT G - 3'. This PCR  
fragment contains a silent mutation that at the same  
time destroys a *Pfl*MI site present at position 1379 of  
the human FSHR cDNA and introduces a *Pvu*I site at the  
same position. ii) *Sac*II 2080 - SENSE: SEQ ID NO:22: 5'  
35 - C CAT CCG CGG AAT GGC CAC TGC TCT TCA GC - 3' and  
vector M13 (-20) PRIMER: SEQ ID NO:23: 5' - GTA AAA CGA  
CGG CCA GT - 3'. This PCR fragment contains a silent



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mutation that at the same time destroys a *Pfl*MI site present at position 2,080 of the human FSHR cDNA and introduces a *Sac*II site at the same position. iii) *Pvu*I 1379 - SENSE: SEQ ID NO:24: 5' - TAT GCG ATC GAC TGG CAA ACT GGG GCA GG - 3' and *Sac*II 2080 - ANTI-SENSE: SEQ ID NO:25: 5' - C ATT CCG CGG ATG GGT GTT GTG GAC AGT G - 3'. PCR fragment that contains two silent mutations that simultaneously change a *Pfl*MI site present at positions 1,379 into a *Pvu*I site and a *Pfl*MI site present at position 2,080 into a *Sac*II site (numbers refer to the nucleotide sequence of the original hFSHR cDNA). PCR fragments originated with oligonucleotide pairs: i) was cut with the restriction enzymes *Bst*EII and *Pvu*I and a 225 bp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure; ii) was cut with the restriction enzymes *Sac*II and *Apa*I and a 700 bp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure; iii) was cut with the restriction enzymes *Pvu*I and *Sac*II and a 140 bp DNA fragment was purified by agarose gel electrophoresis followed by the Mermaid™ procedure. Construct obtained in step # 3 was cut with the restriction enzymes *Bst*EII and *Apa*I and a 2.9 kbp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure. Finally the four purified DNA fragments were ligated together to produce a modified hFSHR construct in pBSSKII(+)*AT* vector.

5) Assembling of the hTHRR amino terminus ectodomain obtained from step # 2 with the modified hFSHR construct obtained from step # 4: the DNA fragment encoding the hTHRR amino terminus from amino acid residue F<sup>55</sup> to L<sup>96</sup> obtained from step # 2 was digested with the restriction enzyme *Bst*EII; the modified pBSSKII(+)*AT*-hFSHR construct obtained from step # 4 was linearized with the restriction enzyme *Sac*I and blunted with T4 DNA polymerase to remove 3'

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overhangs. After enzyme inactivation, blunted linear pBSSKII(+)-hFSHR was cut with the restriction enzyme BstEII. After appropriate DNA fragment purification the two modified DNA fragments (hTHRR Blunt-BstEII ~130 bp long and pBSSKII(+)-hFSHR Blunt-BstEII ~3,800 bp long) generated at this step (# 5) were ligated together to form the intermediate construct pBSSKII(+)-hTHRR/hFSHR. Correctness of the sequence was verified by dideoxynucleotide sequencing method using Circumvent sequencing kit (New England Biolabs, Inc.).

6) Plasmid construct generated at step # 1 (pBSSKII(+)-PROLAC FLAG (EcoRI-HindIII)) was cut with the restriction enzymes HindIII and ApaI and the larger fragment was purified by the GeneClean™ procedure.

Plasmid construct obtained at step # 6 (intermediate pBSSKII(+)-hTHRR/hFSHR) was cut with the restriction enzymes PflMI and ApaI and the resulting ~1,100 bp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure. A pair of oligonucleotides CONNECT-SENSE: SEQ ID NO:26: 5' - AG CTT GAT GCC ACG CTA TGG CCC TAG GTA AGT GAT ATG CCA CCT T - 3'; and CONNECT-ANTI-SENSE: SEQ ID NO:27: 5' - G TGG CAT ATC ACT TAC CTA GGG CCA TAG CGT GGC ATC A - 3' were annealed and purified using the procedure described in step # 1. Annealed CONNECT-SENSE/CONNECT-ANTI-SENSE oligonucleotides were used to adapt the overhang created by HindIII digestion of pBSSKII(+)-PROLAC FLAG with the overhang created by PflMI digestion of intermediate pBSSKII(+)-hTHRR/hFSHR, both mentioned above. Therefore, a ligation of the two DNA fragments purified above was performed using the adaptor CONNECT-SENSE/CONNECT-ANTI-SENSE. This adaptor not only regenerates the two restriction enzyme sites, HindIII and PflMI, but also introduces a second PflMI site between the HindIII and the first PflMI sites. PflMI restriction enzyme recognition sequence is an interrupted palindrome that allows for the directional

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clone of DNA fragments with appropriate overhangs. The two PflMI restriction enzyme sites were created to subclone a synthetic DNA fragment that introduces, in frame with both the prolactin leader sequence/FLAG epitope tag and the chimeric hTHRR/hFSHR sequence described in step # 5, the variable pentapeptide agonist library preceded and followed by hTHRR amino acid residues corresponding to hTHRR residues from L<sup>34</sup> to P<sup>55</sup>. CONNECT-SENSE/CONNECT-ANTI-SENSE adaptor sequence introduces stop codons at each of the three possible reading frames to decrease background levels during the construction of the library. Correctness of the sequence was verified by dideoxynucleotide sequencing method using automatic sequencing.

7) Subcloning of the construct obtained at step # 6 into the mammalian expression vector pcDNA3 and into unmodified pBSSKII(+). Plasmid obtained in step # 6 was digested with EcoRI and NotI, followed by purification of the ~1300 bp DNA insert. pcDNA3 and pBSSKII(+) were both prepared by cutting with EcoRI and NotI. Insert and vectors were ligated. A library created in the vector pcDNA3 is used in transfection experiments using mammalian cells. Fig. 9 shows the plasmid map for the THRR/FSHR construct designated pcDNA3PROLACFLAGhTHRR/hFSHR. A library created in the vector pBSSKII(+) is used for in vitro transcription after linearization with the restriction enzyme NotI. Resulting RNAs are injected into *Xenopus* oocytes.

8) Library construction: three oligonucleotides: coding strand LIBRARY-1: SEQ ID NO:28: 5' - PO<sub>4</sub> - A GAT CCC CGG NNS NNS NNS NNS NNS AAC CCC AAT GAT AAA TAT GAA CCC TT - 3', where N means all four nucleotides and S means either G or C, a degenerate oligonucleotide pool with 2<sup>25</sup> different nucleic acid molecules, encoding 20<sup>5</sup> different pentapeptide sequences; complementary strand LIBRARY-2: SEQ ID NO:29: 5' - PO<sub>4</sub> - CCC GGG ATC TAC C - 3'; and complementary strand LIBRARY-3: SEQ ID NO:30:

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5' - PO<sub>4</sub> - GG TTC ATA TTT ATC - 3' were annealed at a molar ratio of 1 (LIBRARY-1) : 25 (LIBRARY-2) : 25 (LIBRARY-3) in 20 mM Tris-HCl pH 7.6 , 10 mM MgCl<sub>2</sub>, by heating at 95 °C for 5 min and cooling to 4 °C at a rate of 1 °C per 8 min using a thermal controller apparatus. Annealed oligonucleotides were purified by agarose gel electrophoresis using the Mermaid™ kit. pCDNA3 PROLACFLAG-CONNECT-SENSE/CONNECT-ANTI-SENSE-hTHRR/hFSHR or pBSSKII(+) PROLACFLAG-CONNECT-SENSE/CONNECT-ANTI-SENSE-hTHRR/hFSHR from step # 7 were cut with the restriction enzyme PflMI to completion. Purified large fragment of each construct was ligated with the annealed library oligonucleotides at approximately 1:3 molar ratios. The ligated products were ethanol precipitated and redissolved in water for transformation of *E. coli* XL1-Blue cells by electroporation.

Shown below is the nucleotide sequences of  
PROLAC FLAG - CONNECT-SENSE/CONNECT- ANTI-SENSE -  
20 hTHRR/hFSHR and of the assembled LIBRARY-1, -2 and -3  
oligonucleotides to be ligated into the two PflMI sites  
of the insert, substituting for most of the sequence  
corresponding to CONNECT-SENSE/CONNECT-ANTI-SENSE:

```

25      Eco R I AATTCCACC ATG GAC TCC AAG GGC TCG AGC CAG AAG
          GGTGG TAC CTG AGG TTC CCG AGC TCG GTC TTC
              M   D   S   K   G   S   S   Q   K

30      GGA TCT AGA CTG CTG CTG CTG CTG GTG GTG AGC AAC CTG CTG
CCT AGA TCT GAC GAC GAC GAC GAC CAC CAC TCG TTG GAC GAC
    G   S   R   L   L   L   L   L   V   V   S   N   L   L

35      CTG TGC CAG GGC GTC GTG AGC GAC TAC AAG GAC GAC GAC GAC
GAC ACG GTC CCG CAG CAC TCG CTG ATG TTC CTG CTG CTG CTG
    L   C   Q   G   V   V   S   D   Y   K   D   D   D   D

40      A Hind III AG CTT GAT GCC ACG CT Pfl M I A TGG CCC TAG
TTC GA          A CTA CGG TG          C GAT ACC GGG ATC
              K   L   D   A   T   L
          *

45      GTA AGT GAT ATG CCAC C TT Pfl M I T TGG GAG GAT GAG GAG
CAT TCA CTA TAC GG          G AAA ACC CTC CTA CTC CTC
    *          *          F   W   E   D   E   E

50      AAA AAT CAA AGT GGG TTA ACT GAA TAC AGA TTA GTC TCC ATC
TTT TTA CTT TCA CCC AAT TGA CTT ATG TCA AAT CAG AGG TAG
    K   N   E   S   G   L   T   E   Y   R   L   V   S   I

```

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AAT AAA AGC AGT CCT CTT CAA AAA CAA CTT CCT GCA TTC ATC  
 TTA TTT TCG TCA GGA GAA GTT TTT GTT GAA GGA CGT AAG TAG  
 N K S S P L Q K Q L P A F I

5 TCA GAA GAT GCC TCC G Bst E II GT TAC CTG GGG TAC AAC  
 AGT CTT CTA CGG AGG CCA ATG GAC CCC ATG TTG  
 S E D A S G Y L G Y N

10 ATC CTC AGA GTC CTG ATA TGG TTT ATC AGC ATC CTG GCC ATC  
 TAG GAG TCT CAG GAC TAT ACC AAA TAG TCG TAG GAC CGG TAG  
 I L R V L I W F I S I L A I

15 ACT GGG AAC ATC ATA GTG CTA GTG ATC CTA ACT ACC AGC CAA  
 TGA CCC TTG TAG TAT CAC GAT CAC TAG GAT TGA TGG TCG GTT  
 T G N I I V L V I L T T S Q

20 TAT AAA CTC ACA GTC CCC AGG TTC CTT ATG TGC AAC CTG GCC  
 ATA TTT GAG TGT CAG GGG TCC AAG GAA TAC ACG TTG GAC CGG  
 Y K L T V P R F L M C N L A

25 TTT GCT GAT CTC TGC ATT GGA ATC TAC CTG CTG CTC ATT GCA  
 AAA CGA CTA GAG ACG TAA CCT TAG ATG GAC GAC GAG TAA CGT  
 F A D L C I G I Y L L L I A

30 TCA GTT GAT ATC CAT ACC AAG AGC CAA TAT CAC AAC TAT GCG  
 AGT CAA CTA TAG GTA TGG TTC TCG GTT ATA GTG TTG ATA CGC  
 S V D I H T K S Q Y H N Y A

35 AT PvuI C GAC TGG CAA ACT GGG GCA GGC TGT GAT GCT GCT  
 TAG CTG ACC GTT TGA CCC CGT CCG ACA CTA CGA CGA  
 I D W Q T G A G C D A A

40 GGC TTT TTC ACT GTC TTT GCC AGT GAG CTG TCA GTC TAC ACT  
 CCG AAA AAG TGA CAG AAA CGG TCA CTC GAC AGT CAG ATG TGA  
 G F F T V F A S E L S V Y T

45 CTG ACA GCT ATC ACC TTG GAA AGA TGG CAT ACC ATC ACG CAT  
 GAC TGT CGA TAG TGG AAC CTT TCT ACC GTA TGG TAG TGC GTA  
 L T A I T L E R W H T I T H

50 GCC ATG CAG CTG GAC TGC AAG GTG CAG CTC CGC CAT GCT GCC  
 CGG TAC GTC GAC CTG ACG TTC CAC GTC GAG GCG GTA CGA CGG  
 A M Q L D C K V Q L R H A A

55 AGT GTC ATG GTG ATG GGC TGG ATT TTT GCT TTT GCA GCT GCC  
 TCA CAG TAC CAC TAC CCG ACC TAA AAA CGA AAA CGT CGA CGG  
 S V M V M G W I F A F A A A

60 CTC TTT CCC ATC TTT GGC ATC AGC AGC TAC ATG AAG GTG AGC  
 GAG AAA GGG TAG AAA CCG TAG TCG TCG ATG TAC TTC CAC TCG  
 L F P I F G I S S Y M K V S

65 ATC TGC CTG CCC ATG GAT ATT GAC AGC CCT TTG TCA CAG CTG  
 TAG ACG GAC GGG TAC CTA TAA CTG TCG GGA AAC AGT GTC GAC  
 I C L P M D I D S P L S Q L

70 TAT GTC ATG TCC CTC CTT GTG CTC AAT GTC CTG GCC TTT GTG  
 ATA CAG TAC AGG GAG GAA CAC GAG TTA CAG GAC CGG AAA CAC  
 Y V M S L L V L N V L A F V

75 GTC ATC TGT GGC TGC TAT ATC CAC ATC TAC CTC ACA GTG CGG  
 CAG TAG ACA CCG ACG ATA TAG GTG TAG ATG GAG TGT CAC GCC  
 V I C G C Y I H I Y L T V R

80 AAC CCC AAC ATC GTG TCC TCC TCT AGT GAC ACC AGG ATC GCC  
 TTG GGG TTG TAG CAC AGG AGA TCA CTG TGG TCC TAG CGG  
 N P N I V S S S S D T R I A

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```

AAG CGC ATG GCC ATG CTC ATC TTC ACT GAC TTC CTC TGC ATG
TTC GCG TAC CGG TAC GAG TAG AAG TGA CTG AAG GAG ACG TAC
  K   R   M   A   M   L   I   F   T   D   F   L   C   M

5  GCA CCC ATT TCT TTC TTT GCC ATT TCT GCC TCC CTC AAG GTG
   CGT GGG TAA AGA AAG AAA CGG TAA AGA CGG AGG GAG TTC CAC
   A   P   I   S   F   F   A   I   S   A   S   L   K   V

10 CCC CTC ATC ACT GTG TCC AAA GCA AAG ATT CTG CTG GTT CTG
   GGG GAG TAG TGA CAC AGG TTT CGT TTC TAA GAC GAC CAA GAC
   P   L   I   T   V   S   K   A   K   I   L   L   V   L

15 TTT CAC CCC ATC AAC TCC TGT GCC AAC CCC TTC CTC TAT GCC
   AAA GTG GGG TAG TTG AGG ACA CGG TTG GGG AAG GAG ATA CGG
   F   H   P   I   N   S   C   A   N   P   F   L   Y   A

20 ATC TTT ACC AAA AAC TTT CGC AGA GAT TTC TTC ATT CTG CTG
   TAG AAA TGG TTT TTG AAA GCG TCT CTA AAG AAG TAA GAC GAC
   I   F   T   K   N   F   R   R   D   F   F   I   L   L

25 AGC AAG TGT GGC TGC TAT GAA ATG CAA GCC CAA ATT TAT AGG
   TCG TTC ACA CCG ACG ATA CTT TAC GTT CGG GTT TAA ATA TCC
   S   K   C   G   C   Y   E   M   Q   A   Q   I   Y   R

30 ACA GAA ACT TCA TCC ACT GTC CAC AAC ACC CAT CCG C Sac
   TGT CTT TGA AGT AGG TGA CAG GTG TTG TGG GTA GG
   T   E   T   S   S   T   V   H   N   T   H   P

II GG AAT GGC CAC TGC TCT TCA GCT CCC AGA GTC ACC AAT
C GCC TTA CCG GTG ACG AGA AGT CGA GGG TCT CAG TGG TTA
  R   N   G   H   C   S   S   A   P   R   V   T   N

35 GGT TCC ACT TAC ATA CTT GTC CCT CTA AGT CAT TTA GCC CAA
   CCA AGG TGA ATG TAT GAA CAG GGA GAT TCA GTA AAT CGG GTT
   G   S   T   Y   I   L   V   P   L   S   H   L   A   Q

AAC TAA GC Not I
TTG ATT CGCCGG
  N   *

```

40 The DNA sequence (sense strand) is shown in  
 SEQ ID NO:31, with the antisense strand shown in SEQ ID  
 NO:32. The DNA sequence is the sequence that would  
 encode the amino acid sequence (SEQ ID NO:33) of the  
 45 chimeric preprotein that is prolactin signal  
 peptide/FLAG epitope tag/hTHR receptor amino terminus  
 corresponding to amino acid residues 34 to 96 in the  
 native receptor/hFSH receptor. There are three  
 nonsense ("stop") codons (one in each potential reading  
 50 frame) in the middle of the sequence encoding the hTHR  
 receptor amino terminus that are present to prevent  
 translation of this precursor sequence if this sequence  
 persisted, that is remained uncut, during construction  
 of the final library (see below). These "stop" codons,  
 55 therefore, would prevent translation of non-recombinant

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protein. To construct the library, this sequence (SEQ ID NO:31) is cut with PflMI to excise one small DNA fragment flanked by two PflMI restriction sites that is replaced with the following DNA sequences: sense, SEQ ID NO:28; antisense, SEQ ID NO:29 and SEQ ID NO:30) that encodes the pentapeptide library (amino acid SEQ ID NO:42):

```

10  Pfl M I A GAT CCC CGG NNS NNS NNS NNS NNS AAC CCC AAT
      C GAT CTA GGG GCC
      T L D P R X X X X X N P N

      GAT AAA TAT GAA CCC TT Pfl M I
15  CTA TTT ATA CTT GG
      D K Y E P F

```

Modification of the original construct with the intent to create a combinatorial peptide library that expresses random pentapeptides tethered to the seven transmembrane helical bundle of any GPCR already in an "active" or "exposed" form, without the need for cleavage by thrombin. Use of the human follicle stimulating hormone receptor (hFSH-R) as the initial library construction:

In this version of the library, the variable pentapeptide sequence is placed immediately after the prolactin signal peptide. Consequently, the cleavage produced by the signal peptidase that normally occurs during synthesis of type III membrane proteins would expose or "activate" the pentapeptide present at the beginning of the amino terminus, allowing it to interact with the seven transmembrane helical bundle of the GPCR to which it is tethered. The resulting protein sequence of the amino terminus ectodomain that can replace the amino terminus ectodomain of any GPCR is shown in SEQ ID NO:34:

```

40  MDSKGSSQKGSRLLLLLVVSNLLLCQGVVSXXXNPNPKYEFWEDEEKNESGLTEYRLVSIKNS
      SPLQKQLPAFISEDASGYL

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To create this construct it is necessary to perform a small modification in the construct already obtained for the discovery of peptide agonists for the hFSHR using thrombin activation. By silent mutation using PCR method, it is possible to introduce a new PflMI restriction endonuclease cleavage site in the sequence of prolactin signal peptide construct obtained previously at step # 1: PCR using the pair of oligonucleotide primers : complementary strand PflMI

10 SIGNAL: SEQ ID NO:35: 5' - ATC AAG CTT GTC GTC GTC GTC CTT GTA GTC GCT CAC CAC GCC CTG - 3' and vector M13 (-20) PRIMER: SEQ ID NO:23: 5' - GTA AAA CGA CGG CCA GT - 3', using as template the construct pBSSKII(+) PROLAC FLAG - CONNECT-SENSE/CONNECT-ANTI-SENSE - hTHRR/hFSHR.

15 Resulting PCR product is cut with *EcoRI* and *HindIII*. This fragment will substitute the corresponding fragment in pBSSKII(+) PROLAC FLAG - CONNECT-SENSE/CONNECT-ANTI-SENSE - hTHRR/hFSHR, therefore introducing a third PflMI restriction enzyme recognition site at the desired position. See sequence below (sense, SEQ ID NO:36; antisense, SEQ ID NO:37; amino acid, SEQ ID NO:38):

25 *EcoRI* AATTCACC ATG GAC TCC AAG GGC TCG AGC CAG AAG  
GGTGG TAC CTG AGG TTC CCG AGC TCG GTC TTC  
M D S K G S S Q K

30 GGA TCT AGA CTG CTG CTG CTG CTG GTG GTG AGC AAC CTG CTG  
CCT AGA TCT GAC GAC GAC GAC GAC CAC CAC TCG TTG GAC GAC  
G S R L L L L L V V S N L L

35 CTG TGC CAG GGC *PflMI* GTG GTG AGC GAC TAC AAG GAC GAC  
GAC ACG GTC CCG CAC CAC TCG CTG ATG TTC CTG CTG  
L C Q G V V S D Y K D D

40 GAC GAC A *HindIII* AG CTT GAT GCC ACG CT *PflMI* A TGG  
CTG CTG TTC GA A CTA CGG TG C GAT ACC  
D D K L D A T L L W

45 CCC TAG GTA AGT GAT ATG CCAC C TT *PflMI* T TGG GAG GAT  
GGG ATC CAT TCA CTA TAC GGTG G AAA ACC CTC CTA  
P \* \* \* F W E D

GAG GAG AAA AAT GAA AGT GGG TTA ACT GAA TAC AGA TTA GTC TCC  
CTC CTC TTT TTA CTT TCA CCC AAT TGA CTT ATG TCA AAT CAG AGG  
E E K N E S G L T E Y R L V S

ATC AAT AAA AGC AGT CCT CTT CAA AAA CAA CTT CCT GCA TTC ATC



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TAG TTA TTT TCG TCA GGA GAA GTT TTT GTT GAA GGA CGT AAG TAG  
 I N K S S P L Q K Q L P A F I

5 TCA GAA GAT GCC TCC G Bst E II GT TAC CTG GGG TAC AAC  
 AGT CTT CTA CGG AGG CCA ATG GAC CCC ATG TTG  
 S E D A S G Y L G Y N

10 ATC CTC AGA GTC CTG ATA TGG TTT ATC AGC ATC CTG GCC ATC  
 TAG GAG TCT CAG GAC TAT ACC AAA TAG TCG TAG GAC CGG TAG  
 I L R V L I W F I S I L A I

15 ACT GGG AAC ATC ATA GTG CTA GTG ATC CTA ACT ACC AGC CAA  
 TGA CCC TTG TAG TAT CAC GAT CAC TAG GAT TGA TGG TCG GTT  
 T G N I I V L V I L T T S Q

20 TAT AAA CTC ACA GTC CCC AGG TTC CTT ATG TGC AAC CTG GCC  
 ATA TTT GAG TGT CAG GGG TCC AAG GAA TAC ACG TTG GAC CGG  
 Y K L T V P R F L M C N L A

25 TTT GCT GAT CTC TGC ATT GGA ATC TAC CTG CTG CTC ATT GCA  
 AAA CGA CTA GAG ACG TAA CCT TAG ATG GAC GAC GAG TAA CGT  
 F A D L C I G I Y L L L I A

30 TCA GTT GAT ATC CAT ACC AAG AGC CAA TAT CAC AAC TAT GCG  
 AGT CAA CTA TAG GTA TGG TTC TCG GTT ATA GTG TTG ATA CGC  
 S V D I H T K S Q Y H N Y A

35 AT PvuI C GAC TGG CAA ACT GGG GCA GGC TGT GAT GCT GCT  
 TAG CTG ACC GTT TGA CCC CGT CCG ACA CTA CGA CGA  
 I D W Q T G A G C D A A

40 GGC TTT TTC ACT GTC TTT GCC AGT GAG CTG TCA GTC TAC ACT  
 CCG AAA AAG TGA CAG AAA CGG TCA CTC GAC AGT CAG ATG TGA  
 G F F T V F A S E L S V Y T

45 CTG ACA GCT ATC ACC TTG GAA AGA TGG CAT ACC ATC ACG CAT  
 GAC TGT CGA TAG TGG AAC CTT TCT ACC GTA TGG TAG TGC GTA  
 L T A I T L E R W H T I T H

50 GCC ATG CAG CTG GAC TGC AAG GTG CAG CTC CGC CAT GCT GCC  
 CGG TAC GTC GAC CTG ACG TTC CAC GTC GAG GCG GTA CGA CGG  
 A M Q L D C K V Q L R H A A

55 AGT GTC ATG GTG ATG GGC TGG ATT TTT GCT TTT GCA GCT GCC  
 TCA CAG TAC CAC TAC CCG ACC TAA AAA CGA AAA CGT CGA CGG  
 S V M V M G W I F A F A A A

60 CTC TTT CCC ATC TTT GGC ATC AGC AGC TAC ATG AAG GTG AGC  
 GAG AAA GGG TAG AAA CCG TAG TCG TCG ATG TAC TTC CAC TCG  
 L F P I F G I S S Y M K V S

65 ATC TGC CTG CCC ATG GAT ATT GAC AGC CCT TTG TCA CAG CTG  
 TAG ACG GAC GGG TAC CTA TAA CTG TCG GGA AAC AGT GTC GAC  
 I C L P M D I D S P L S Q L

70 TAT GTC ATG TCC CTC CTT GTG CTC AAT GTC CTG GCC TTT GTG  
 ATA CAG TAC AGG GAG GAA CAC GAG TTA CAG GAC CGG AAA CAC  
 Y V M S L L V L N V L A F V

75 GTC ATC TGT GGC TGC TAT ATC CAC ATC TAC CTC ACA GTG CGG  
 CAG TAG ACA CCG ACG ATA TAG GTG TAG ATG GAG TGT CAC GCC  
 V I C G C Y I H I Y L T V R

80 AAC CCC AAC ATC GTG TCC TCC TCT AGT GAC ACC AGG ATC GCC  
 TTG GGG TTG TAG CAC AGG AGG AGA TCA CTG TGG TCC TAG CGG  
 N P N I V S S S S D T R I A

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AAG CGC ATG GCC ATG CTC ATC TTC ACT GAC TTC CTC TGC ATG  
 TTC GCG TAC CGG TAC GAG TAG AAG TGA CTG AAG GAG ACG TAC  
 K R M A M L I F T D F L C M  
 5 GCA CCC ATT TCT TTC TTT GCC ATT TCT GCC TCC CTC AAG GTG  
 CGT GGG TAA AGA AAG AAA CGG TAA AGA CGG AGG GAG TTC CAC  
 A P I S F F A I S A S L K V  
 10 CCC CTC ATC ACT GTG TCC AAA GCA AAG ATT CTG CTG GTT CTG  
 GGG GAG TAG TGA CAC AGG TTT CGT TTC TAA GAC GAC CAA CGG  
 P L I T V S K A K I L L V L  
 15 TTT CAC CCC ATC AAC TCC TGT GCC AAC CCC TTC CTC TAT GCC  
 AAA GTG GGG TAG TTG AGG ACA CGG TTG GGG AAG GAG ATA CGG  
 F H P I N S C A N P F L Y A  
 20 ATG TTT ACC AAA AAC TTT CGC AGA GAT TTC TTC ATT CTG CTG  
 TAG AAA TGG TTT TTG AAA GCG TCT CTA AAG AAG TAA GAC GAC  
 I F T K N F R R D F F I L L  
 25 AGC AAG TGT GGC TGC TAT GAA ATG CAA GCC CAA ATT TAT AGG  
 TCG TTC ACA CCG ACG ATA CTT TAC GTT CGG GTT TAA ATA TCC  
 S K C G C Y E M Q A Q I Y R  
 30 ACA GAA ACT TCA TCC ACT GTC CAC AAC ACC CAT CCG C *Sac*  
 TGT CTT TGA AGT AGG TGA CAG GTG TTG TGG GTA GG  
 T E T S S T V H N T H P  
 35 GGT TCC ACT TAC ATA CTT GTC CCT CTA AGT CAT TTA GCC CAA  
 CCA AGG TGA ATG TAT GAA CAG GGA GAT TCA GTA AAT CGG GTT  
 G S T Y I L V ? L S H L A Q  
 40 AAC TAA GC Not I  
 TTG ATT CGCCGG  
 N \*

Two of the three LIBRARY oligonucleotides are  
 also need to be modified as follows: coding strand  
 LIBRARY-4: SEQ ID NO:39: 5' - PO<sub>4</sub> - GTG GTG AGC NNS NNS  
 NNS NNS NNS AAC CCC AAT GAT AAA TAT GAA CCC TT - 3';  
 45 and complementary strand LIBRARY-5: SEQ ID NO:40: 5' -  
 PO<sub>4</sub> - GCT CAC CAC GCC - 3'.

The nucleotide sequence of the assembled  
 LIBRARY-4, -5 and -3 oligonucleotides to be ligated into  
 the two *Pfl*MI sites of the insert, substituting for the  
 50 sequence corresponding to FLAG - CONNECT-SENSE/  
 CONNECT-ANTI-SENSE is shown below (sense, SEQ ID NO:39;  
 antisense, SEQ ID NO:40 and SEQ ID NO:30; amino acid,  
 SEQ ID NO:41):

55

*Pfl* M I GTG GTG AGC NNS NNS NNS NNS NNS AAC CCC AAT GAT

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```

          CCG CAC CAC TCG          CTA
            G  V  V  S    X  X  X  X  X  N  P  N  D

5  AAA TAT GAA CCC TT Pfl M I
   TTT ATA CTT GG
     K  Y  E  P  F

```

The DNA sequence is shown in SEQ ID NO:39. This sequence is the same as SEQ ID NO:31 except that it has a "silent" mutation that creates another PflMI restriction site. The DNA sequence (SEQ ID NO:39) is the sequence that would encode the amino acid sequence (SEQ ID NO:41) of the chimeric preprotein that is prolactin signal peptide/FLAG epitope tag/hTHR receptor amino terminus corresponding to amino acid residues 34 to 96 in the native receptor/hFSH receptor sequence from amino acid residue 361 to 694 of the native hFSH receptor. There are three nonsense ("stop") codons (one in each potential reading frame) in the middle of the sequence encoding the hTHR receptor amino terminus that are present to prevent translation of this precursor sequence if this sequence persisted, that is remained uncut, during construction of the final library. These "stop" codons, therefore, would prevent translation of non-recombinant protein. To construct the library, this sequence (SEQ ID NO:39) is cut with PflMI to excise two small DNA fragments flanked by two PflMI restriction sites that is replaced with the following DNA sequences: sense, SEQ ID NO:39; antisense, SEQ ID NO:40 and SEQ ID NO:30) that encodes the pentapeptide library (SEQ ID NO:41).

## EXAMPLE II

### Peptide Agonists of hCTR

Fig. 6 illustrates the putative two-dimensional topology of a human calcitonin receptor (hCTR). The top of the diagram represents the extracellular (EC) space, the middle portion represents the transmembrane (TM) domain, and the bottom portion represents the intracellular (IC) space. Each circle

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represents a single amino acid residue designated by the single letter code. The residues specifically referred to in this application are numbered with regard to hCTR-2. Bold lines demarcate the 3 isoforms:

5 hCTR-1 - all residues; hCTR-2 - missing the 16 amino acids between R174 and S175; and hCTR-3 - missing residues 1-47.

To discover small peptides that can serve as agonists for hCTR, a combinatorial peptide library was constructed that expresses random pentapeptides

10 tethered to the seven TM helical bundle of hCTR. A pentapeptide library was chosen based on the fact that TRH is a tripeptide that is blocked at both ends (3+2 (for block) =5) and the resulting number of clones is

15 workable. The constructed library contains all 20 natural amino acids at each of the five positions and therefore has a complexity of  $20^5 = 3.2 \times 10^6$  possible combinations.

To this end, the complementary DNA (cDNA)

20 sequence that normally encodes hCTR's N-terminal EC domain is substituted by a DNA sequence that encodes the thrombin receptor's N-terminal ectodomain. The chimeric ThrR/hCTR has the variable pentapeptide sequence substituting for the native peptide sequence

25 that is normally unmasked by thrombin action and constitutes the ThrR peptide agonist, but it retains thrombin binding sequences and the thrombin-specific cleavage site. Therefore, the N-terminus of expressed receptors are cleaved by thrombin at the appropriate

30 location exposing a new N-terminus that is made of the variable pentapeptide segment of the library tethered to the remainder of hCTR, that is, in a position that in the native ThrR allows it to serve as an agonist.

To monitor for cell surface expression and

35 for efficient cleavage by thrombin of the N-terminal end of the chimeric receptors, the FLAG epitope is positioned proximally to the thrombin cleavage site.

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Abs that recognize ThrR N-terminus distal to the position corresponding to the library are also used. Consequently, chimeric receptors expressed on the cell surface are detectable by the appropriate use of both  
5 Abs before thrombin treatment, but only with Abs against the distal part after thrombin treatment. This confirms cell-surface expression and adequacy of thrombin generation of potential agonists.

The cDNA sequence encoding the new N-terminus  
10 of the chimeric ThrR/hCTR, consisting of a prolactin leader or signal peptide, followed by the FLAG epitope, followed by the N-terminus of the mature human ThrR and the pentapeptide library is constructed by gene synthesis. It consists of a DNA segment of  
15 approximately 300 base pairs encoding 100 amino acids that is ligated in frame through an appropriate restriction endonuclease cleavage site that is in the synthetic hCTR-2 cDNA at a position encoding the amino acids that constitute the transition between the  
20 N-terminus and the first TM domain. After ligation into a mammalian expression vector, *Escherichia coli* is transformed by electroporation and the transformants are subdivided into pools whose maximal workable complexity is determined according to the efficiency of  
25 expression and/or sensitivity of the detection system.

The success of expression cloning strategies, such as the one of the subject invention, is dependent on the reporter (or detection) system used. An amplified reporter system is used in accordance with  
30 the subject invention which is based on the second messenger systems triggered by hCTR. hCTR is a GPCR that upon activation in COS-1 cells couples through the G protein,  $G_s$ , to the enzyme adenylyl cyclase, causing an increase in intracellular concentrations of cyclic  
35 AMP (cAMP) and through  $G_q$  to the enzyme phospholipase C causing increases in inositol 1,4,5-trisphosphate (IP3), which causes a rise in intracellular free  $Ca^{2+}$ ,

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and 1,2-diacylglycerol (DAG) (Nussenzveig et al. 1994). cAMP activates cAMP- dependent protein kinase (PKA), an important intracellular regulator. One PKA substrate is a transcription factor known as cAMP-response element binding protein, CREB, that when activated binds and activates transcription by promoters that contain regulatory sequences known as cAMP-response elements (CREs). A similar cascade initiated by IP<sub>3</sub>, DAG and an elevation of cytoplasmic Ca<sup>2+</sup> that involves other protein kinases triggers gene induction through other motifs using other transcription factors, such as the fos-jun- AP-1 system. This type of reporter system is able to detect basal as well as CT-stimulated activation of the wild type hCTR by using a reporter plasmid containing a minimal promoter of human c-fos gene into which a CREB binding motif (Montminy et al. 1990) was engineered driving transcription of the gene for the enzyme luciferase (pCRE/LUC), whose activity is easily detected by a chemiluminescent reaction. Unfortunately, the use of the enzyme activity of the luciferase reporter system requires the preparation of cell extracts and, therefore, monitors induction in a population of cells. To be able to measure a single positive in a very large number of negatives from a library, a single cell assay is needed. Two different assays are used so as to improve the likelihood of identifying positive clones.

One assay is based on gene induction in COS-1 cells.  $\beta$ -galactosidase is used as a reporter gene in transfected COS-1 cells. This assay takes advantage of the amplification of the enzyme activity of the reporter, with an easily determined color reaction as endpoint, and of the over-expression of receptors with tethered agonists in COS-1 cells because of replication of the plasmids introduced. These experiments were performed by co-transfecting portions of the library and CRE/ $\beta$ -galactosidase or AP-1/ $\beta$ -galactosidase

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constructs into COS-1 cells so as to amplify expression by plasmid replication using the simian virus-40 origin of replication in the vector. This enhances the signal/noise ratio substantially. Both promoter types are used as hCTR-2 transduces signals by both pathways in COS-1 cells. The signal is further increased because the construct used has a nuclear localization signal ligated to the  $\beta$ -galactosidase that allows the protein to concentrate in the nucleus (Hersh et al. 1995). Single clones that exhibit activation of chimeric ThrR/hCTR after thrombin addition to cleave the N-terminus and expose the tethered agonist, as measured by increased color reaction, are isolated using sib selection, which consists of successive subdivision and amplification of positive pools of clones. The optimal time after thrombin addition to measure the reporter gene activity is determined, as this involves a prolonged response on gene induction and the kinetics of this response vary with different activators (receptors) and in different cells. The optimal time will likely be 4 hrs as that was optimal for SCT stimulation in COS-1 cells co-transfected with hCTR-2 and luciferase under the control of a CAMP-responsive promoter (CRE-LUC). Even though hCTR-2 exhibits constitutive activity, gene expression can be further activated with agonist.

The second reporter system uses *Xenopus laevis* oocytes. This system, which was used to clone the TRH receptor cDNA (Straub et al. 1990), is dependent on coupling to  $G_q$ . In this assay system, individual oocytes are injected with RNA that was transcribed in vitro from pools of plasmids from the library. After one to three days (the optimal time for responsiveness is determined in preliminary experiments with hCTR-2), the responsiveness of the oocytes to thrombin is measured. Thrombin acutely activates receptors in those oocytes expressing chimeric

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constructs in which the peptide can serve as a tethered agonist. Receptor activation is monitored by acute effects on a chloride current or on stimulation of radiocalcium efflux. Both endpoints are rapid, amplified processes in oocytes. Single clones that exhibit activation of chimeric ThrR/hCTR after thrombin addition are isolated using sib selection. Using both reporter systems allows the determination of whether rapid or more prolonged effects yield better signal-to-noise ratios.

Other reporting systems may also be useful in the cloning strategy, such as an immunofluorescence/immunocytochemical approach in COS-1 cells that also relies on gene induction. Commercially available anti-GFP Abs (Clontech) or anti- $\beta$ -galactosidase Abs (Promega Biotech, Inc.) can be used to identify transfected COS-1 cells in which ectopic gene expression has been induced. Or, a plasmid can be constructed in which CRE or AP-1 drives expression of a cell-surface protein to which Abs are available, such as nerve growth factor receptor (Johnson et al. 1986), and FACS sorting can be used to identify positive cells. Alternatively, a rapid effect of activation of the ThrR/hCTR in COS-1 cells can be monitored. Stimulation of an acute elevation in cytoplasmic  $Ca^{2+}$  using Fluo-3 (Molecular Probes) and fluorescence microscopy can be measured. Fluorescent calcium indicators (Geras Raaka and Gershengorn 1987) can be used.

The strategy in accordance with the subject invention for the design of the library suits the purpose for which it is intended to be used because: i) It removes the putative EC N-terminal domain of hCTR to which intact, native CT binds with high affinity. Without hCTR's N-terminus, the possibility of finding a peptide that acts indirectly through hCTR's N-terminus is eliminated. ii) It produces receptors that activate



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only upon addition of thrombin. This allows for receptors to be active only during the experimental period, avoiding the cellular counterregulatory mechanisms associated with prolonged stimulation (desensitization, down regulation), which can attenuate the detecting signal. iii) A tethered agonist increases the local effective concentration of the ligand enormously. This reduces the possibility that peptide antagonists, if present in the same pool of an untethered peptide library, could interfere with the detection of peptide agonists.

### EXAMPLE III

#### Peptide Negative Antagonists of a GPCR of HHV-8

This example relates to a newly described GPCR that is encoded in the genome of human herpesvirus-8 (HHV-8) (or Kaposi's sarcoma-associated herpesvirus - KSHV) (Cesarman et al. 1996), which is a virus that was first identified in Kaposi's sarcoma (KS) tissues from patients with AIDS and has now been found in KS tissues from human immunodeficiency virus (HIV)-negative patients, in tissues from patients with Castleman's disease and in some B-cell lymphomas (Chang et al. 1994). This receptor is referred to as HHV8 GPCR. The objective of this example is to identify peptides that are high affinity negative antagonists of this constitutively active HHV8 GPCR. A constitutively active receptor is a receptor that exhibits agonist-independent signalling activity (Lefkowitz et al. 1993). Negative antagonists (or inverse agonists) are compounds that are capable of inhibiting the signalling activity of a constitutively active receptor (Schutz and Freissmuth 1992). Neutral antagonists inhibit the action of agonists but do not affect agonist-independent activity. Neutral antagonists would inhibit signalling by HHV8 GPCR when it is activated by natural or synthetic agonists, for

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example, interleukin-8 (IL-8) that has been shown to activate HHV8 GPCR in *Xenopus laevis* oocytes, whereas negative antagonists would inhibit "basal" signalling by HHV8 GPCR.

5           The HHV8 GPCR is a protein of 342 amino acids that has the features of a GPCR including seven hydrophobic, putative transmembrane-spanning domains (Cesarman et al. 1996). Fig. 7 illustrates the putative two-dimensional topology of HHV8 GPCR in the  
10 cell-surface membrane. The top of the diagram represents the extracellular space (E), the middle portion represents the transmembrane (TM) domain and the bottom portion represents the intracellular space (C). Each circle represents an amino acid residue  
15 designated by the single letter code. The HHV8 GPCR is a receptor that signals via the phosphoinositide-inositol 1,4,5-trisphosphate-calcium cascade (Berridge 1993).

20   *Discovery of peptide negative antagonists of HHV8 GPCR*

It is now appreciated that receptors can attain an active conformation in the absence of agonist and manifest constitutive, that is, agonist-independent activity (Lefkowitz et al. 1993). This has led to  
25 renewed acceptance of the concept that receptors can change conformation spontaneously and oscillate between active and inactive states (for review, see Leff 1995). Some drugs, termed negative antagonists or inverse agonists, appear capable of constraining receptors in  
30 an inactive state (Samama et al. 1994). Negative antagonism is demonstrated when a drug binds to a receptor that exhibits constitutive activity and reduces this activity. It is important to discover agents that exhibit negative antagonistic properties  
35 toward HHV8 GPCR to use in exploring the role of HHV8 GPCR during HHV-8 infection in studies in cells in tissue culture and in intact animals.

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The subject invention provides a strategy for discovery of small peptide negative antagonists of HHV8 GPCR. A tethered, combinatorial library is used to clone pentapeptides that are negative antagonists of HHV8 GPCR. A pentapeptide library is chosen based on the fact small peptides are effective negative antagonists and the number of clones is workable. The library contains all 20 natural amino acids at each of the five positions and therefore has a complexity of  $20^5 = 3.2 \times 10^6$  possible combinations. This approach is chosen because although there is a good deal of information available regarding IL-8 binding (see above), little is known regarding the specific interactions between IL-8 and IL-8Rs that cause activation (Leong et al. 1994). In fact, this is true for GPCRs in general (Van Rhee and Jacobson 1996). Moreover, there is even less known about specific interactions that may inactivate a constitutively active receptor (Schutz and Freissmuth 1992). Thus, insufficient information is available to "rationally" design small peptides with negative antagonist activities. Thus, discovery of negative antagonist peptides for HHV8 GPCR may best be accomplished by using combinatorial peptide libraries. With this approach, 3.2 million random peptides of five amino acids in length are tested for activity and those that inactivate HHV8 GPCRs are identified by sib selection.

*Discovery of high affinity, specific pentapeptide negative antagonists of HHV8 GPCR*

To discover small peptides that can serve as negative antagonists (or inverse agonists) for HHV8 GPCR, a combinatorial peptide library is constructed that expresses random pentapeptides tethered to the seven TM helical bundle of HHV8 GPCR. This strategy is based on the conclusion that one (or several) pentapeptides will interact with the TM bundle or

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extracellular loops, or both of HHV8 GPCR in a manner similar to that by which other small peptide antagonists interact with other GPCRs, such as receptors for opioid peptides (Costa and Herz 1989; Costa et al. 1992) and bradykinin (Leeb-Lundberg et al. 1994), and by a similar mechanism inactivate HHV8 GPCR.

A pentapeptide library is chosen based on the fact that peptides of this size have been shown to be negative antagonists of other GPCRs and the resulting number of clones is workable. The library contains all 20 natural amino acids at each of the five positions and, therefore, has a complexity of  $20^5 = 3.2 \times 10^6$  possible compounds. The library is constructed by taking the cDNA sequence of HHV8 GPCR and substituting the sequence that normally encodes HHV8 GPCR's N-terminal extracellular domain by a DNA sequence that encodes the N-terminal ectodomain of the thrombin receptor (ThrR) from just after the activating peptide to the beginning of TM-1; that is, the sequence of the native ThrR from its N-terminus up to and including its activating peptide, Ser-Phe-Leu-Leu-Arg-Asn (SEQ ID NO:43:SFLLRN), is deleted. The chimeric ThrR/HHV8 GPCR primary amino acid sequence begins at its N-terminus with the variable pentapeptide sequence ("library"), which is substituting for SFLLRN, followed by the ThrR amino terminal sequence distal to the SFLLRN sequence (from immediately after SFLLRN to the beginning of TM-1) followed by the HHV8GPCR sequence from the beginning of TM-1 to the carboxyl end (Fig. 8). The distal N-terminal sequence of the ThrR is chosen rather than that of HHV8 GPCR because this sequence allows the pentapeptide library sequences on each ThrR/HHV8 GPCR chimera to be directed into the remainder of the receptor as the exposed N-terminal peptide of ThrR is guided into the receptor's "body". The major difference is that the pentapeptide library is the N-terminus of the ThrR/HHV8 GPCR tethered to the

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remainder of the receptor, that is, in a position that in the native ThrR allows it to serve as an agonist but allows it in the chimeric receptor to serve as a negative antagonist. No cleavage is necessary to expose the N-terminus pentapeptide sequence. Therefore, the N-terminus of expressed receptors are random pentapeptides that can act as negative antagonists with regard to the constitutive activity of HHV8 GPCRs as soon as the chimeric receptor is expressed. The library is constructed without the need to cleave off a "blocking" sequence in order to expose the pentapeptide because it is desirable for the pentapeptide to inactivate the chimeric receptor as soon as it is expressed on the cell surface. Thus, monitoring is for inactivation of a "basal" signalling activity of the chimeric ThrR/HHV8 GPCR.

Fig. 8 shows the putative topology of the chimera ThrR/HHV8 GPCR as it is predicted to be in the cell surface membrane of transfected COS-1 cells. The top of the diagram represents the extracellular space (E), the middle portion represents the transmembrane (TM) domain and the bottom portion represents the intracellular space (C). The first five filled circles represent individual amino acids that are part of the pentapeptide library; each filled circle represents 20 amino acids. The seventy unfilled circles represent the individual amino acid residues of the native ThrR sequence from just after the activating peptide (SFLLRN) to the beginning of TM-1. Each circle with a letter in it represents an amino acid residue designated by the single letter code of HHV8 GPCR.

To monitor for cell surface expression of the chimeric receptors, antibodies to the extracellular domain of HHV8 GPCR are used, specifically antibodies to the large extracellular loop 2.

The cDNA sequence encoding the new N-terminus of the chimeric ThrR/HHV8 GPCR, consisting of a

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prolactin leader (or signal) peptide, which is cleaved after directing the protein to the cell surface membrane, followed by the pentapeptide library and the distal sequence of the N-terminus of ThrR is constructed by gene synthesis. It consists of a DNA segment of approximately 210 base pairs encoding 70 amino acids that are ligated in frame through an appropriate restriction endonuclease cleavage site that is created in the HHV8 GPCR cDNA at a position encoding the amino acids that constitute the transition between the N-terminus and the first TM domain. After ligation into a mammalian expression vector, *Escherichia coli* is transformed by electroporation and the transformants are subdivided into pools whose maximal workable complexity is determined according to the efficiency of mammalian cell transfection and/or sensitivity of the detection system(s).

The success of expression cloning strategies, such as the one of the subject invention, is dependent on the reporter (or detection) system. An amplified reporter system is used in accordance with the subject invention which is based on the second messenger system triggered by HHV8 GPCR. HHV8 GPCR is a GPCR that in COS-1 cells appears to couple through a G protein to the enzyme phospholipase C causing generation of the second messengers inositol 1,4,5-trisphosphate ( $IP_3$ ), which causes a rise in intracellular free  $Ca^{2+}$ , and 1,2-diacylglycerol, which activates protein kinase C (Nussenzveig et al. 1994). Activated protein kinase C triggers gene induction through specific motifs using transcription factors, such as the fos-jun-AP-1 system (Deutsch et al. 1990; Schadow et al. 1992). This reporter system works in COS-1 cells since constitutive activity of HHV8 GPCR is detected using a reporter plasmid containing a minimal promoter of the human c-fos gene into which a AP-1 binding motif is engineered driving transcription of the gene for the

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enzyme luciferase (pAP-1/LUC), whose activity is detected by a chemiluminescent reaction.

Unfortunately, the use of the enzyme activity of the luciferase reporter system requires the preparation of cell extracts and, therefore, monitors induction in a population of cells. To be able to identify receptors that are turned off by negative antagonistic activity of the tethered pentapeptide, a single "hit" in a very large number of negatives needs to be measured. Therefore, a single cell assay is needed. For the reporter, luciferase is replaced with  $\beta$ -galactosidase that can be readily measured in individual cells.

A two-reporter system was devised for discovery of negative antagonists that use gene induction in COS-1 cells.  $\beta$ -galactosidase is used as a reporter enzyme in transfected COS-1 cells. This assay takes advantage of the amplification of the enzyme activity of the reporter, with an easily determined color reaction as endpoint, and of the over-expression of receptors with tethered negative antagonists in COS-1 cells because of replication of the plasmids introduced. These experiments are performed by co-transfecting portions of the plasmid library and a plasmid encoding AP-1/ $\beta$ -galactosidase constructs into COS-1 cells so as to amplify expression by plasmid replication using the simian virus-40 origin of replication in the vector. This enhances the signal/noise ratio substantially. The signal is further increased because the construct used has a nuclear localization signal ligated to the  $\beta$ -galactosidase that allows the protein to concentrate in the nucleus (Hersh et al. 1995). The construct containing  $\beta$ -galactosidase with a nuclear localization signal was shown to express in the nucleus of transfected COS-1 cells. Single clones that exhibit negative antagonistic activity, as measured by decreased color reaction, are isolated using sib

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selection, which consists of successive subdivision and amplification of positive pools of clones. The optimal time after transfection to assay  $\beta$ -galactosidase activity is determined empirically as this involves a  
5 prolonged response on gene induction and the kinetics of this response vary with different activators (receptors) and in different cells.

A second reporter gene is used to identify cells that have been transfected and are expressing  
10 foreign proteins to distinguish them from cells that have not been transfected. This is a crucial distinction for this approach because differentiation between cells that have the capacity to express the specific reporter gene but are not (or in which  
15 expression has been diminished) because transcription has been inhibited, from cells that are not expressing the reporter gene because they are not transfected, is necessary. Because the  $\beta$ -galactosidase activity is expressed in the nucleus, it has a different  
20 localization than the nonspecific reporter of transfection. The nonspecific reporter of transfection is a construct containing a mutant of the human placental alkaline phosphatase gene (Tate et al. 1990) that is targeted to the cytoplasm under the control of  
25 a cytomegalovirus promoter; this promoter is not affected by HHV8 GPCR and is active in all transfected cells. Thus, one can monitor for 3 types of cells: 1) cells in which  $\beta$ -galactosidase is expressed at high levels in the nucleus and alkaline phosphatase is  
30 expressed in the cytoplasm - these are transfected cells that do not express receptors that contain a peptide that has negative antagonistic activity because expression of  $\beta$ -galactosidase is induced by the constitutive signalling activity of HHV8 GPCR; 2) cells  
35 in which  $\beta$ -galactosidase is not expressed in the nucleus and alkaline phosphatase is not expressed in the cytoplasm - these are cells that have not been



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transfected; and 3) cells in which  $\beta$ -galactosidase is not expressed (or is expressed at low levels in the nucleus) and alkaline phosphatase is expressed in the cytoplasm - these are transfected cells that express  
5 receptors that contain a peptide that has negative antagonistic activity.

Other reporting systems may also be useful in the cloning strategy, such as the yeast bioassay system discussed above or an immunofluorescence/  
10 immunocytochemical approach in COS-1 cells that also relies on gene induction. Commercially available anti- $\beta$ -galactosidase antibodies (Promega Biotech, Inc.) can be used to identify transfected COS-1 cells in which ectopic gene expression has been modulated. Or,  
15 a plasmid can be constructed in which AP-1 drives the expression of a cell-surface protein to which Abs are available, such as the nerve growth factor receptor (Johnson et al. 1986).

The strategy devised in the design of the  
20 library suits the purpose for which it is intended to be used, because a tethered negative antagonist increases the local effective concentration of the ligand enormously. This also reduces the possibility that neutral antagonists or agonists, if present in the  
25 same pool of an untethered peptide library, could interfere with the detection of peptide negative antagonists.

Although preferred embodiments have been  
30 depicted and described in detail herein, it will be apparent to those of ordinary skill in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore  
35 considered to be within the scope of the invention as defined in the claims which follow.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Cornell Research Foundation, Inc.
- (ii) TITLE OF INVENTION: STRATEGY TO CLONE DRUGS FOR G  
PROTEIN COUPLED RECEPTORS
- (iii) NUMBER OF SEQUENCES: 44
- (iv) CORRESPONDENCE ADDRESS:
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  - (C) CITY: Rochester
  - (D) STATE: New York
  - (E) COUNTRY: USA
  - (F) ZIP: 14603
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/795,876
  - (B) FILING DATE: 06-FEB-1997
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Weyand, Karla M.
  - (B) REGISTRATION NUMBER: 40,223
  - (C) REFERENCE/DOCKET NUMBER: 19603/1281
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 716-263-1508
  - (B) TELEFAX: 716-263-1600

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 63 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Leu Asp Ala Thr Leu Asp Pro Arg Ser Phe Leu Leu Arg Asn Pro Asn
1           5           10           15
Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu Glu Lys Asn Glu Ser Gly
20           25           30
Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn Lys Ser Ser Pro Leu Gln
35           40           45
Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp Ala Ser Gly Tyr Leu
50           55           60

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 436 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
1           5           10           15
Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr
20           25           30
Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Asp Pro Arg Xaa Xaa
35           40           45
Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu
50           55           60
Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn
65           70           75           80
Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp
85           90           95
Ala Ser Gly Tyr Leu Gly Tyr Asn Ile Leu Arg Val Leu Ile Trp Phe
100          105          110
Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu Val Ile Leu
115          120          125
Thr Thr Ser Gln Tyr Lys Leu Thr Val Pro Arg Phe Leu Met Cys Asn
130          135          140
Leu Ala Phe Ala Asp Leu Cys Ile Gly Ile Tyr Leu Leu Leu Ile Ala
145          150          155          160
Ser Val Asp Ile His Thr Lys Ser Gln Tyr His Asn Tyr Ala Ile Asp
165          170          175

```

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Trp Gln Thr Gly Ala Gly Cys Asp Ala Ala Gly Phe Phe Thr Val Phe  
 180 185 190  
 Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Ala Ile Thr Leu Glu Arg  
 195 200 205  
 Trp His Thr Ile Thr His Ala Met Gln Leu Asp Cys Lys Val Gln Leu  
 210 215 220  
 Arg His Ala Ala Ser Val Met Val Met Gly Trp Ile Phe Ala Phe Ala  
 225 230 235 240  
 Ala Ala Leu Phe Pro Ile Phe Gly Ile Ser Ser Tyr Met Lys Val Ser  
 245 250 255  
 Ile Cys Leu Pro Met Asp Ile Asp Ser Pro Leu Ser Gln Leu Tyr Val  
 260 265 270  
 Met Ser Leu Leu Val Leu Asn Val Leu Ala Phe Val Val Ile Cys Gly  
 275 280 285  
 Cys Tyr Ile His Ile Tyr Leu Thr Val Arg Asn Pro Asn Ile Val Ser  
 290 295 300  
 Ser Ser Ser Asp Thr Arg Ile Ala Lys Arg Met Ala Met Leu Ile Phe  
 305 310 315 320  
 Thr Asp Phe Leu Cys Met Ala Pro Ile Ser Phe Phe Ala Ile Ser Ala  
 325 330 335  
 Ser Leu Lys Val Pro Leu Ile Thr Val Ser Lys Ala Lys Ile Leu Leu  
 340 345 350  
 Val Leu Phe His Pro Ile Asn Ser Cys Ala Asn Pro Phe Leu Tyr Ala  
 355 360 365  
 Ile Phe Thr Lys Asn Phe Arg Arg Asp Phe Phe Ile Leu Leu Ser Lys  
 370 375 380  
 Cys Gly Cys Tyr Glu Met Gln Ala Gln Ile Tyr Arg Thr Glu Thr Ser  
 385 390 395 400  
 Ser Thr Val His Asn Thr His Pro Arg Asn Gly His Cys Ser Ser Ala  
 405 410 415  
 Pro Arg Val Thr Asn Gly Ser Thr Tyr Ile Leu Val Pro Leu Ser His  
 420 425 430  
 Leu Ala Gln Asn  
 435

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu  
 1 5 10 15  
 Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser  
 20 25 30

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Tyr Lys Asp Asp Asp Asp Lys  
 1 5

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Xaa Xaa Xaa Xaa Xaa  
 1 5

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATTCCACCA TGGACTCCAA GGGCTCGAGC CAGAAGGGAT CTAGACTGCT

50

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 50 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGCAGCAGT CTAGATCCCT TCTGGCTCGA GCCCTTGGAG TCCATGGTGG

50

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 46 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCTGCTGCTG GTGGTGAGCA ACCTGCTGCT GTGCCAGGGC GTCGTG

46

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 46 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCTCACGAC GCCCTGGCAC AGCAGCAGGT TGCTCACCAC CAGCAG

46

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## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCGACTACA AGGACGACGA CGACAAGCTT CCTGCCTTTT

40

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGAAAAGGCA GGAAGCTTGT CGTCGTCGTC CTTGTAGT

38

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 54 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATGCCACCT TTTGGGAGGA TGAGGAGAAA AATGAAAGTG GGTAACTGA ATAC

54

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## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 56 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGAAGAGGAC TGCTTTTATT GATGGAGACT AATCTGTATT CAGTTAACCC ACTTTC

56

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 55 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CAATAAAAGC AGTCCTCTTC AAAAACAAC TCTGCATTC ATCTCAGAAG ATGCC

55

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCAGGTAAC CGGAGGCATC TTCTGAGATG AATGCAAG

38



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## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGAAGGTTAC CTGGGGTACA ACATCCTCAG AGTCC

35

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCACGCGGCC GCTTAGTTTT GGGCTAAATG ACTTAGAGG

39

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AATTCGGCTT

10

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## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCCG

5

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAGTCGATCG CATAGTTGTG ATATTGGCTC

30

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AACAGCTATG ACCATG

16

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## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCATCCGCGG AATGGCCACT GCTCTTCAGC

30

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTAAAACGAC GGCCAGT

17

## (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TATGCGATCG ACTGGCAAAC TGGGGCAGG

29

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## (2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CATTCCGCGG ATGGGTGTTG TGGACAGTG

29

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGCTTGATGC CACGCTATGG CCCTAGGTAA GTGATATGCC ACCTT

45

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTGGCATATC ACTTACCTAG GGCCATAGCG TGGCATCA

38

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## (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGATCCCCGG AACCCCAATG ATAAATATGA ACCCTT

36

## (2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 13 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCGGGGATCT AGC

13

## (2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 14 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGTTCATATT TATC

14

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## (2) INFORMATION FOR SEQ ID NO:31:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1300 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AATTCCACCA TGGACTCCAA GGGCTCGAGC CAGAAGGGAT CTAGACTGCT GCTGCTGCTG	60
GTGGTGAGCA ACCTGCTGCT GTGCCAGGGC GTCGTGAGCG ACTACAAGGA CGACGACGAC	120
AAGCTTGATG CCACGCTATG GCCCTAGGTA AGTGATATGC CACCTTTTGG GAGGATGAGG	180
AGAAAAATGA AAGTGGGTGA ACTGAATACA GATTAGTCTC CATCAATAAA AGCAGTCCTC	240
TTCAAAAACA ACTTCCTGCA TTCATCTCAG AAGATGCCCTC CGGTTACCTG GGTACAACA	300
TCCTCAGAGT CCTGATATGG TTTATCAGCA TCCTGGCCAT CACTGGAAC ATCATAGTGC	360
TAGTGATCCT AACTACCAGC CAATATAAAC TCACAGTCCC CAGGTTCTTT ATGTCAACC	420
TGGCCTTTGC TGATCTCTGC ATTGGAATCT ACCTGCTGCT CATTGCATCA GTTGATATCC	480
ATACCAAGAG CCAATATCAC AACTATGCGA TCGACTGGCA AACTGGGGCA GGCTGTGATG	540
CTGCTGGCTT TTTCACTGTC TTTGCCAGTG AGCTGTCAGT CTACACTCTG ACAGCTATCA	600
CCTTGGAAG ATGGCATAAC ATCAGCATG CCATGCAGCT GGACTGCAAG GTGCAGCTCC	660
GCCATGCTGC CAGTGTCATG GTGATGGGCT GGATTTTTC TTTGCAGCT GCCCTCTTTC	720
CCATCTTTGG CATCAGCAGC TACATGAAGG TGAGCATCTG CCTGCCCATG GATATTGACA	780
GCCCTTTGTC ACAGCTGTAT GTCATGTCCC TCCTTGTGCT CAATGTCCTG GCCTTTGTGG	840
TCATCTGTGG CTGCTATATC CACATCTACC TCACAGTGCG GAACCCCAAC ATCGTGTCTT	900
CCTCTAGTGA CACCAGGATC GCCAAGCGCA TGGCCATGCT CATCTTCACT GACTTCCTCT	960
GCATGGCACC CATTTCTTTC TTTGCCATTT CTGCCTCCCT CAAGGTGCCC CTCATCACTG	1020
TGTCCAAAGC AAAGATTCTG CTGGTTCTGT TTCACCCCAT CAACTCCTGT GCCAACCCCT	1080
TCCTCTATGC CATCTTTACC AAAAATTTT GCAGAGATTT CTTATTCTG CTGAGCAAGT	1140
GTGGCTGCTA TGAAATGCAA GCCCAAATTT ATAGGACAGA AACTTCATCC ACTGTCCACA	1200
ACACCCATCC GCGGAATGGC CACTGCTCTT CAGCTCCAG AGTCACCAAT GGTCCACTT	1260
ACATACTTGT CCCTCTAAGT CATTTAGCCC AAAACTAAGC	1300

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## (2) INFORMATION FOR SEQ ID NO:32:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1298 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGCCGCTTAG TTTTGGGCTA AATGACTTAG AGGGACAAGT ATGTAAGTGG AACCATTGGT	60
GACTCTGGGA GCTGAAGAGC AGTGGCCATT CCGCGGATGG GTGTTGTGGA CAGTGGATGA	120
AGTTTCTGTC CTATAAATTT GGGCTTGCAT TTCATAGCAG CCACACTTGC TCAGCAGAAT	180
GAAGAAATCT CTGCGAAAGT TTTTGGTAAA GATGGCATAG AGGAAGGGGT TGGCACAGGA	240
GTTGATGGGG TGAAACAGAA CCAGCAGAAT CTTTGCTTTG GACACAGTGA TGAGGGGCAC	300
CTTGAGGGAG GCAGAAATGG CAAAGAAAGA AATGGGTGCC ATGCAGAGGA AGTCAGTGAA	360
GATGAGCATG GCCATGCGCT TGGCGATCCT GGTGTCACTA GAGGAGGACA CGATGTTGGG	420
GTTCCGCACT GTGAGGTAGA TGTGGATATA GCAGCCACAG ATGACCACAA AGGCCAGGAC	480
ATTGAGCACA AGGAGGGACA TGACATACAG CTGTGACAAA GGGCTGTCAA TATCCATGGG	540
CAGGCAGATG CTCACCTTCA TGTAGCTGCT GATGCCAAAG ATGGGAAAGA GGGCAGCTGC	600
AAAAGCAAAA ATCCAGCCCA TCACCATGAC ACTGGCAGCA TGGCGGAGCT GCACCTTGCA	660
GTCCAGCTGC ATGGCATGCG TGATGGTATG CCATCTTTCC AAGGTGATAG CTGTCAGAGT	720
GTAGACTGAC AGCTCACTGG CAAAGACAGT GAAAAAGCCA GCAGCATCAC AGCCTGCCCC	780
AGTTTGCCAG TCGATCGCAT AGTTGTGATA TTGGCTCTTG GTATGGATAT CAACTGATGC	840
AATGAGCAGC AGGTAGATTC CAATGCAGAG ATCAGCAAAG GCCAGGTTGC ACATAAGGAA	900
CCTGGGGACT GTGAGTTTAT ATTGGCTGGT AGTTAGGATC ACTAGCACTA TGATGTTCCT	960
AGTGATGGCC AGGATGCTGA TAAACCATAT CAGGACTCTG AGGATGTTGT ACCCCAGGTA	1020
ACCGGAGGCA TCTTCTGAGA TGAATGCAGG AAGTTGTTTT TGAAGAGGAC TGCTTTTATT	1080
GATGGAGACT AACTGTATT CAGTTAACCC ACTTTCATTT TTCTCCTCAT CCTCCCAAAA	1140
GGGCATATCA CTTACCTAGG GCCATAGCGT GGCATCAAGC TTGTCGTCGT CGTCTTGTA	1200
GTCGCTCAGC ACGCCCTGGC ACAGCAGCAG GTTGCTCACC ACCAGCAGCA GCAGCAGTCT	1260
AGATCCCTTC TGGCTCGAGC CCTTGGAGTC CATGGTGG	1298

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## (2) INFORMATION FOR SEQ ID NO:33:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 420 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
 1          5          10          15
Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr
 20          25          30
Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Phe Trp Glu Asp Glu
 35          40          45
Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn
 50          55          60
Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp
 65          70          75
Ala Ser Gly Tyr Leu Gly Tyr Asn Ile Leu Arg Val Leu Ile Trp Phe
 85          90          95
Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu Val Ile Leu
100          105          110
Thr Thr Ser Gln Tyr Lys Leu Thr Val Pro Arg Phe Leu Met Cys Asn
115          120          125
Leu Ala Phe Ala Asp Leu Cys Ile Gly Ile Tyr Leu Leu Leu Ile Ala
130          135          140
Ser Val Asp Ile His Thr Lys Ser Gln Tyr His Asn Tyr Ala Ile Asp
145          150          155
Trp Gln Thr Gly Ala Gly Cys Asp Ala Ala Gly Phe Phe Thr Val Phe
165          170          175
Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Ala Ile Thr Leu Glu Arg
180          185          190
Trp His Thr Ile Thr His Ala Met Gln Leu Asp Cys Lys Val Gln Leu
195          200          205
Arg His Ala Ala Ser Val Met Val Met Gly Trp Ile Phe Ala Phe Ala
210          215          220
Ala Ala Leu Phe Pro Ile Phe Gly Ile Ser Ser Tyr Met Lys Val Ser
225          230          235
Ile Cys Leu Pro Met Asp Ile Asp Ser Pro Leu Ser Gln Leu Tyr Val
245          250          255

```



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Met Ser Leu Leu Val Leu Asn Val Leu Ala Phe Val Val Ile Cys Gly  
 260 265 270

Cys Tyr Ile His Ile Tyr Leu Thr Val Arg Asn Pro Asn Ile Val Ser  
 275 280 285

Ser Ser Ser Asp Thr Arg Ile Ala Lys Arg Met Ala Met Leu Ile Phe  
 290 295 300

Thr Asp Phe Leu Cys Met Ala Pro Ile Ser Phe Phe Ala Ile Ser Ala  
 305 310 315 320

Ser Leu Lys Val Pro Leu Ile Thr Val Ser Lys Ala Lys Ile Leu Leu  
 325 330 335

Val Leu Phe His Pro Ile Asn Ser Cys Ala Asn Pro Phe Leu Tyr Ala  
 340 345 350

Ile Phe Thr Lys Asn Phe Arg Arg Asp Phe Phe Ile Leu Leu Ser Lys  
 355 360 365

Cys Gly Cys Tyr Glu Met Gln Ala Ile Ile Tyr Arg Thr Glu Thr Ser  
 370 375 380

Ser Thr Val His Asn Thr His Pro Arg Asn Gly His Cys Ser Ser Ala  
 385 390 395 400

Pro Arg Val Thr Asn Gly Ser Thr Tyr Ile Leu Val Pro Leu Ser His  
 405 410 415

Leu Ala Gln Asn  
 420

## (2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 85 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu  
 1 5 10 15

Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Xaa Xaa  
 20 25 30

Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu  
 35 40 45

Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn  
 50 55 60

Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp  
 65 70 75 80

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Ala Ser Gly Tyr Leu  
85

## (2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 45 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ATCAAGCTTG TCGTCGTCGT CCTTGTAGTC GCTCACCACG CCCTG

45

## (2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1300 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AATTCCACCA TGGACTCCAA GGGCTCGAGC CAGAAGGGAT CTAGACTGCT GCTGCTGCTG	60
GTGGTGAGCA ACCTGCTGCT GTGCCAGGGC GTGGTGAGCG ACTACAAGGA CGACGACGAC	120
AAGCTTGATG CCACGCTATG GCCCTAGGTA AGTGATATGC CACCTTTTGG GAGGATGAGG	180
AGAAAAATGA AAGTGGGTTA ACTGAATACA GATTAGTCTC CATCAATAAA AGCAGTCCTC	240
TTCAAAAACA ACTTCCTGCA TTCATCTCAG AAGATGCCTC CGGTTACCTG GGGTACAACA	300
TCCTCAGAGT CCTGATATGG TTTATCAGCA TCCTGGCCAT CACTGGGAAC ATCATAGTGC	360
TAGTGATCCT AACTACCAGC CAATATAAAC TCACAGTCCC CAGGTTCTTT ATGTGCAACC	420
TGGCCTTTGC TGATCTCTGC ATTGGAATCT ACCTGCTGCT CATTGCATCA GTTGATATCC	480
ATACCAAGAG CCAATATCAC AACTATGCGA TCGACTGGCA AACTGGGGCA GGCTGTGATG	540
CTGCTGGCTT TTCACTGTC TTTGCCAGTG AGCTGTCACT CTACACTCTG ACAGCTATCA	600
CCTTGGAAAG ATGGCATAAC ATCAGCATG CCATGCAGCT GGACTGCAAG GTGCAGCTCC	660
GCCATGCTGC CAGTGTCTATG GTGATGGGCT GGATTTTTCG TTTTGCAGCT GCCCTCTTTC	720
CCATCTTTGG CATCAGCAGC TACATGAAGG TGAGCATCTG CCTGCCCATG GATATTGACA	780

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GCCCTTTGTC ACAGCTGTAT GTCATGTCCC TCCTTGCTCT CAATGTCCTG GCCTTTGTGG 840  
 TCATCTGTGG CTGCTATATC CACATCTACC TCACAGTGCG GAACCCCAAC ATCGTGTCTT 900  
 CCTCTAGTGA CACCAGGATC GCCAAGCGCA TGGCCATGCT CATCTTCACT GACTTCCTCT 960  
 GCATGGCACC CATTCTTTTC TTGCCATTT CTGCCTCCCT CAAGGTGCCC CTCATCACTG 1020  
 TGTCCAAAGC AAAGATTCTG CTGGTTCTGT TTCACCCCAT CAACTCCTGT GCCAACCCTT 1080  
 TCCTCTATGC CATCTTTACC AAAAATTTT GCAGAGATT CTTCATTCTG CTGAGCAAGT 1140  
 GTGGCTGCTA TGAAATGCAA GCCCAAATTT ATAGGACAGA AACTTCATCC ACTGTCCACA 1200  
 ACACCCATCC GCGGAATGGC CACTGCTCTT CAGCTCCAG AGTCACCAAT GGTTCACCTT 1260  
 ACATACTTGT CCCTCTAAGT CATTTAGCCC AAACTAAGC 1300

## (2) INFORMATION FOR SEQ ID NO:37:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1300 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGCCGCTTAG TTTTGGGCTA AATGACTTAG AGGGACAAGT ATGTAAGTGG AACCATTGGT 60  
 GACTCTGGGA GCTGAAGAGC AGTGGCCATT CCGCGGATGG GTGTTGTGGA CAGTGGATGA 120  
 AGTTTCTGTC CTATAAATTT GGGCTTGCAT TTCATAGCAG CCACACTTGC TCAGCAGAAT 180  
 GAAGAAATCT CTGCGAAAGT TTTTGGTAAA GATGGCATAG AGGAAGGGGT TGGCACAGGA 240  
 GTTGATGGGG TGAAACAGAA CCAGCAGAAT CTTTGCTTTG GACACAGTGA TGAGGGGCAC 300  
 CTTGAGGGAG GCAGAAATGG CAAAGAAAGA AATGGGTGCC ATGCAGAGGA AGTCAGTGAA 360  
 GATGAGCATG GCCATGCGCT TGGCGATCCT GGTGTCTACTA GAGGAGGACA CGATGTTGGG 420  
 GTTCCGCACT GTGAGGTAGA TGTGGATATA GCAGCCACAG ATGACCACAA AGGCCAGGAC 480  
 ATTGAGCACA AGGAGGGACA TGACATACAG CTGTGACAAA GGGCTGTCAA TATCCATGGG 540  
 CAGGCAGATG CTCACCTTCA TGTAGCTGCT GATGCCAAAG ATGGGAAAGA GGGCAGCTGC 600  
 AAAAGCAAAA ATCCAGCCCA TCACCATGAC ACTGGCAGCA TGGCGAGCT GCACCTTGCA 660  
 GTCCAGCTGC ATGGCATGCG TGATGGTATG CCATCTTCC AAGGTGATAG CTGTCAGAGT 720  
 GTAGACTGAC AGCTCACTGG CAAAGACAGT GAAAAAGCCA GCAGCATCAC AGCCTGCCCC 780  
 AGTTTGCCAG TCGATCGCAT AGTTGTGATA TTGCTCTTG GTATGCATAT CAACTGATGC 840  
 AATGAGCAGC AGGTAGATTC CAATGCAGAG ATCAGCAAAG GCCAGGTTGC ACATAAGGAA 900

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CCTGGGGACT GTGAGTTTAT ATTGGCTGGT AGTTAGGATC ACTAGCACTA TGATGTTCCC 960  
 AGTGATGGCC AGGATGCTGA TAAACCATAT CAGGACTCTG AGGATGTTGT ACCCCAGGTA 1020  
 ACCGGAGGCA TCTTCTGAGA TGAATGCAGG AAGTTGTTTT TGAAGAGGAC TGCTTTTATT 1080  
 GATGGAGACT AAACGTGATT CAGTTAACCC ACTTTCATTT TTCTCCTCAT CCTCCCAAAA 1140  
 GGTGGCATAT CACTTACCTA GGGCCATAGC GTGGCATCAA GCTTGTCGTC GTCGTCCTTG 1200  
 TAGTCGCTCA CCACGCCCTG GCACAGCAGC AGGTTGCTCA CCACCAGCAG CAGCAGCAGT 1260  
 CTAGATCCCT TCTGGCTCGA GCCCTTGGAG TCCATGGTGG 1300

## (2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 423 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu  
 1 5 10 15  
 Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr  
 20 25 30  
 Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Leu Trp Pro Phe Trp  
 35 40 45  
 Glu Asp Glu Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val  
 50 55 60  
 Ser Ile Asn Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile  
 65 70 75 80  
 Ser Glu Asp Ala Ser Gly Tyr Leu Gly Tyr Asn Ile Leu Arg Val Leu  
 85 90 95  
 Ile Trp Phe Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu  
 100 105 110  
 Val Ile Leu Thr Thr Ser Gln Tyr Lys Leu Thr Val Pro Arg Phe Leu  
 115 120 125  
 Met Cys Asn Leu Ala Phe Ala Asp Leu Cys Ile Gly Ile Tyr Leu Leu  
 130 135 140  
 Leu Ile Ala Ser Val Asp Ile His Thr Lys Ser Gln Tyr His Asn Tyr  
 145 150 155 160  
 Ala Ile Asp Trp Gln Thr Gly Ala Gly Cys Asp Ala Ala Gly Phe Phe  
 165 170 175

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Thr Val Phe Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Ala Ile Thr  
 180 185 190  
 Leu Glu Arg Trp His Thr Ile Thr His Ala Met Gln Leu Asp Cys Lys  
 195 200 205  
 Val Gln Leu Arg His Ala Ala Ser Val Met Val Met Gly Trp Ile Phe  
 210 215 220  
 Ala Phe Ala Ala Ala Leu Phe Pro Ile Phe Gly Ile Ser Ser Tyr Met  
 225 230 235 240  
 Lys Val Ser Ile Cys Leu Pro Met Asp Ile Asp Ser Pro Leu Ser Gln  
 245 250 255  
 Leu Tyr Val Met Ser Leu Leu Val Leu Asn Val Leu Ala Phe Val Val  
 260 265 270  
 Ile Cys Gly Cys Tyr Ile His Ile Tyr Leu Thr Val Arg Asn Pro Asn  
 275 280 285  
 Ile Val Ser Ser Ser Ser Asp Thr Arg Ile Ala Lys Arg Met Ala Met  
 290 295 300  
 Leu Ile Phe Thr Asp Phe Leu Cys Met Ala Pro Ile Ser Phe Phe Ala  
 305 310 315 320  
 Ile Ser Ala Ser Leu Lys Val Pro Leu Ile Thr Val Ser Lys Ala Lys  
 325 330 335  
 Ile Leu Leu Val Leu Phe His Pro Ile Asn Ser Cys Ala Asn Pro Phe  
 340 345 350  
 Leu Tyr Ala Ile Phe Thr Lys Asn Phe Arg Arg Asp Phe Phe Ile Leu  
 355 360 365  
 Leu Ser Lys Cys Gly Cys Tyr Glu Met Gln Ala Gln Ile Tyr Arg Thr  
 370 375 380  
 Glu Thr Ser Ser Thr Val His Asn Thr His Pro Arg Asn Gly His Cys  
 385 390 395 400  
 Ser Ser Ala Pro Arg Val Thr Asn Gly Ser Thr Tyr Ile Leu Val Pro  
 405 410 415  
 Leu Ser His Leu Ala Gln Asn  
 420

## (2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GTGGTGAGCA ACCCCAATGA TAAATATGAA CCCTT

35

## (2) INFORMATION FOR SEQ ID NO:40:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GCTCACCACG CC

12

## (2) INFORMATION FOR SEQ ID NO:41:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Gly	Val	Val	Ser	Xaa	Xaa	Xaa	Xaa	Xaa	Asn	Pro	Asn	Asp	Lys	Tyr	Glu
1				5					10					15	

Pro Phe

## (2) INFORMATION FOR SEQ ID NO:42:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Thr Leu Asp Pro Arg Xaa Xaa Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr  
 1 5 10 15  
 Glu Pro Phe

## (2) INFORMATION FOR SEQ ID NO:43:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Ser Phe Leu Leu Arg Asn  
 1 5

## (2) INFORMATION FOR SEQ ID NO:44:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 429 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu  
 1 5 10 15  
 Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr  
 20 25 30  
 Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Asp Pro Arg Xaa Xaa  
 35 40 45  
 Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu  
 50 55 60  
 Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn  
 65 70 75 80  
 Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp  
 85 90 95

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Ala Ser Gly Tyr Leu Val Leu Tyr Tyr Leu Ala Ile Val Gly His Ser  
 100 105 110  
 Leu Ser Ile Phe Thr Leu Val Ile Ser Leu Gly Ile Phe Val Phe Phe  
 115 120 125  
 Arg Ser Leu Gly Cys Gln Arg Val Thr Leu His Lys Asn Met Phe Leu  
 130 135 140  
 Thr Tyr Ile Leu Asn Ser Met Ile Ile Ile Ile His Leu Val Glu Val  
 145 150 155 160  
 Val Pro Asn Gly Glu Leu Val Arg Arg Asp Pro Val Ser Cys Lys Ile  
 165 170 175  
 Leu His Phe Phe His Gln Tyr Met Met Ala Cys Asn Tyr Phe Trp Met  
 180 185 190  
 Leu Cys Glu Gly Ile Tyr Leu His Thr Leu Ile Val Val Ala Val Phe  
 195 200 205  
 Thr Glu Lys Gln Arg Leu Arg Trp Tyr Tyr Leu Leu Gly Trp Gly Phe  
 210 215 220  
 Pro Leu Val Pro Thr Thr Ile His Ala Ile Thr Arg Ala Val Tyr Phe  
 225 230 235 240  
 Asn Asp Asn Cys Trp Leu Ser Val Glu Thr His Leu Leu Tyr Ile Ile  
 245 250 255  
 His Gly Pro Val Met Ala Ala Leu Val Val Asn Phe Phe Phe Leu Leu  
 260 265 270  
 Asn Ile Val Arg Val Leu Val Thr Lys Met Arg Glu Thr His Glu Ala  
 275 280 285  
 Glu Ser His Met Tyr Leu Lys Ala Val Lys Ala Thr Met Ile Leu Val  
 290 295 300  
 Pro Leu Leu Gly Ile Gln Phe Val Val Phe Pro Trp Arg Pro Ser Asn  
 305 310 315 320  
 Lys Met Leu Gly Lys Ile Tyr Asp Tyr Val Met His Ser Leu Ile His  
 325 330 335  
 Phe Gln Gly Phe Phe Val Ala Thr Ile Tyr Cys Phe Cys Asn Asn Glu  
 340 345 350  
 Val Gln Thr Thr Val Lys Arg Gln Trp Ala Gln Phe Lys Ile Gln Trp  
 355 360 365  
 Asn Gln Arg Trp Gly Arg Arg Pro Ser Asn Arg Ser Ala Arg Ala Ala  
 370 375 380  
 Ala Ala Ala Ala Glu Ala Gly Asp Ile Pro Ile Tyr Ile Cys His Gln  
 385 390 395 400  
 Glu Leu Arg Asn Glu Pro Ala Asn Asn Gln Gly Glu Glu Ser Ala Glu  
 405 410 415  
 Ile Ile Pro Leu Asn Ile Ile Glu Gln Glu Ser Ser Ala  
 420 425



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**WHAT IS CLAIMED IS:**

1. A method of identifying peptide agonists or negative antagonists of a G protein coupled receptor of interest, said method comprising:

expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell; and

monitoring said cell to determine whether the peptide is an agonist or negative antagonist of said G protein coupled receptor of interest.

2. The method of claim 1 for identifying a peptide agonist wherein expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises:

preparing a G protein coupled receptor construct for identifying a peptide agonist, the G protein coupled receptor construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which is a peptide agonist for activating said self-activating receptor; and

a nucleic acid molecule encoding the peptide of the peptide library inserted into said second amino terminus and replacing said deleted portion;

introducing the G protein coupled receptor construct into a cell;

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allowing said cell to express said G protein coupled receptor encoded thereby; and

exposing said cell to a ligand of said self-activating receptor, wherein said ligand cleaves said G protein coupled receptor construct to expose said inserted peptide of said peptide library.

3. The method of claim 2 wherein said introducing comprises injecting said G protein coupled receptor construct into said cell.

4. The method of claim 2 wherein said introducing comprises transformation of said cell with an expression vector, said expression vector comprising said G protein coupled receptor construct.

5. The method of claim 1 for identifying a peptide negative antagonist wherein said G protein coupled receptor of interest is a constitutively active G protein coupled receptor and wherein expressing a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises:

preparing a constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist, the constitutively active G protein coupled receptor construct comprising:

a nucleic acid molecule encoding a constitutively active G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding said constitutively active G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-

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activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and

a nucleic acid molecule encoding the peptide of the peptide library inserted into said second amino terminus and replacing said deleted portion;

introducing the constitutively active G protein coupled receptor construct into a cell; and

allowing said cell to express said constitutively active G protein coupled receptor encoded thereby.

6. The method of claim 5 wherein said introducing comprises injecting said constitutively active G protein coupled receptor construct into said cell.

7. The method of claim 5 wherein said introducing comprises transformation of said cell with an expression vector, said expression vector comprising said constitutively active G protein coupled receptor construct.

8. The method of claim 1 for identifying a peptide agonist wherein expressing a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises:

preparing a G protein coupled receptor construct for identifying a peptide agonist, the G protein coupled receptor construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid

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molecule encoding said G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-activating receptor as well as any amino acids positioned terminally to the peptide agonist; and

a nucleic acid molecule encoding the peptide of the peptide library inserted into said second amino terminus and replacing said deleted portion;

introducing the G protein coupled receptor construct into a cell; and

allowing said cell to express said G protein coupled receptor encoded thereby.

9. The method of claim 8 wherein said introducing comprises injecting said G protein coupled receptor construct into said cell.

10. The method of claim 8 wherein said introducing comprises transformation of said cell with an expression vector, said expression vector comprising said G protein coupled receptor construct.

11. The method of claim 1 wherein said G protein coupled receptor signals through an ion channel pathway and wherein said monitoring comprises detecting levels of said ion within said cell.

12. The method of claim 11 wherein said ion channel pathway is a calcium channel.

13. The method of claim 12 wherein said cell is a *Xenopus* oocyte and wherein said monitoring comprises voltage clamp analysis.

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14. The method of claim 1 wherein said G protein coupled receptor signals through a cyclic adenosine monophosphate pathway and wherein said monitoring comprises detecting levels of cyclic adenosine monophosphate within said cell.

15. A G protein coupled receptor construct for identifying a peptide agonist of the G protein coupled receptor, the construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which is a peptide agonist for activating said self-activating receptor; and

a nucleic acid molecule encoding a peptide of a peptide library inserted into said second amino terminus and replacing said deleted portion.

16. The G protein coupled receptor construct of claim 15 wherein said self-activating receptor is a thrombin receptor.

17. The G protein coupled receptor construct of claim 16 wherein said second amino terminus of a nucleic acid molecule encoding a thrombin receptor encodes an amino acid sequence as shown in SEQ ID NO:1, and wherein amino acid residues 9 to 13 of SEQ ID NO:1 are the portion which is a peptide agonist for said thrombin receptor.

18. The G protein coupled receptor construct of claim 15 wherein the G protein coupled receptor is a human calcitonin receptor.

19. The G protein coupled receptor construct of claim 18 wherein said construct has an amino acid sequence as shown in SEQ ID NO:44, and wherein amino acid residues 47 to 51 of SEQ ID NO:44 are the peptide of a peptide library, amino acid residues 1 to 101 of SEQ ID NO:44 are the second amino terminus, and amino acid residues 102 to 429 of SEQ ID NO:44 are the nucleic acid molecule encoding the human calcitonin receptor with said first amino terminus deleted.

20. The G protein coupled receptor construct of claim 15 wherein the G protein coupled receptor is a human follicle stimulating hormone receptor.

21. The G protein coupled receptor construct of claim 20 wherein said construct has an amino acid sequence as shown in SEQ ID NO:2, and wherein amino acid residues 47 to 51 of SEQ ID NO:2 are the peptide of a peptide library, amino acid residues 39 to 101 of SEQ ID NO:2 are the second amino terminus, and amino acid residues 102 to 436 of SEQ ID NO:2 are the nucleic acid molecule encoding the human follicle stimulating hormone receptor with said first amino terminus deleted.

22. A cell comprising the G protein coupled receptor construct of claim 15.

23. The cell of claim 22 wherein the cell is a *Xenopus* oocyte.

24. An expression vector comprising the G protein coupled receptor construct of claim 15.

25. The expression vector of claim 24 wherein said expression vector is selected from the group consisting of a plasmid and a virus.



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26. A cell comprising the expression vector of claim 24.

27. A constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist of the constitutively active G protein coupled receptor, the construct comprising:  
a nucleic acid molecule encoding a constitutively active G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a constitutively active G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and

a nucleic acid molecule encoding a peptide of a peptide library inserted into said second amino terminus and replacing said deleted portion.

28. The constitutively active G protein receptor construct of claim 27 wherein said self-activating receptor is a thrombin receptor.

29. The constitutively active G protein coupled receptor construct of claim 28 wherein said second amino terminus of a nucleic acid molecule encoding a thrombin receptor encodes an amino acid sequence as shown in SEQ ID NO:1, and wherein amino acid residues 9 to 13 of SEQ ID NO:1 are the portion which is a peptide agonist for said thrombin receptor.

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30. A cell comprising the constitutively active G protein coupled receptor construct of claim 27.

31. The cell of claim 30 wherein the cell is a *Xenopus* oocyte.

32. The cell of claim 30 wherein the cell is a yeast cell.

33. An expression vector comprising the constitutively active G protein coupled receptor construct of claim 27.

34. The expression vector of claim 33 wherein said expression vector is selected from the group consisting of a plasmid and a virus.

35. A cell comprising the expression vector of claim 34.

36. A G protein coupled receptor construct for identifying a peptide agonist of the G protein coupled receptor, the construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and

a nucleic acid molecule encoding a peptide of a peptide library inserted into said second amino terminus and replacing said deleted portion.

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37. The G protein receptor construct of claim 36 wherein said self-activating receptor is a thrombin receptor.

38. The G protein coupled receptor construct of claim 37 wherein said second amino terminus of a nucleic acid molecule encoding a thrombin receptor encodes an amino acid sequence as shown in SEQ ID NO:1, and wherein amino acid residues 9 to 13 of SEQ ID NO:1 are the portion which is a peptide agonist for said thrombin receptor.

39. The G protein coupled receptor construct of claim 36 wherein the G protein coupled receptor is a human calcitonin receptor.

40. The G protein coupled receptor construct of claim 39 wherein said construct has an amino acid sequence as shown in SEQ ID NO:44, and wherein amino acid residues 47 to 51 of SEQ ID NO:44 are the peptide of a peptide library, amino acid residues 1 to 101 of SEQ ID NO:44 are the second amino terminus, and amino acid residues 102 to 429 of SEQ ID NO:44 are the nucleic acid molecule encoding the human calcitonin receptor with said first amino terminus deleted.

41. The G protein coupled receptor construct of claim 36 wherein the G protein coupled receptor is a human follicle stimulating hormone receptor.

42. The G protein coupled receptor construct of claim 41 wherein said construct has an amino acid sequence as shown in SEQ ID NO:2, and wherein amino acid residues 47 to 51 of SEQ ID NO:2 are the peptide of a peptide library, amino acid residues 39 to 101 of SEQ ID NO:2 are the second amino terminus, and amino acid residues 102 to 436 of SEQ ID NO:2 are the nucleic

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acid molecule encoding the human follicle stimulating hormone receptor with said first amino terminus deleted.

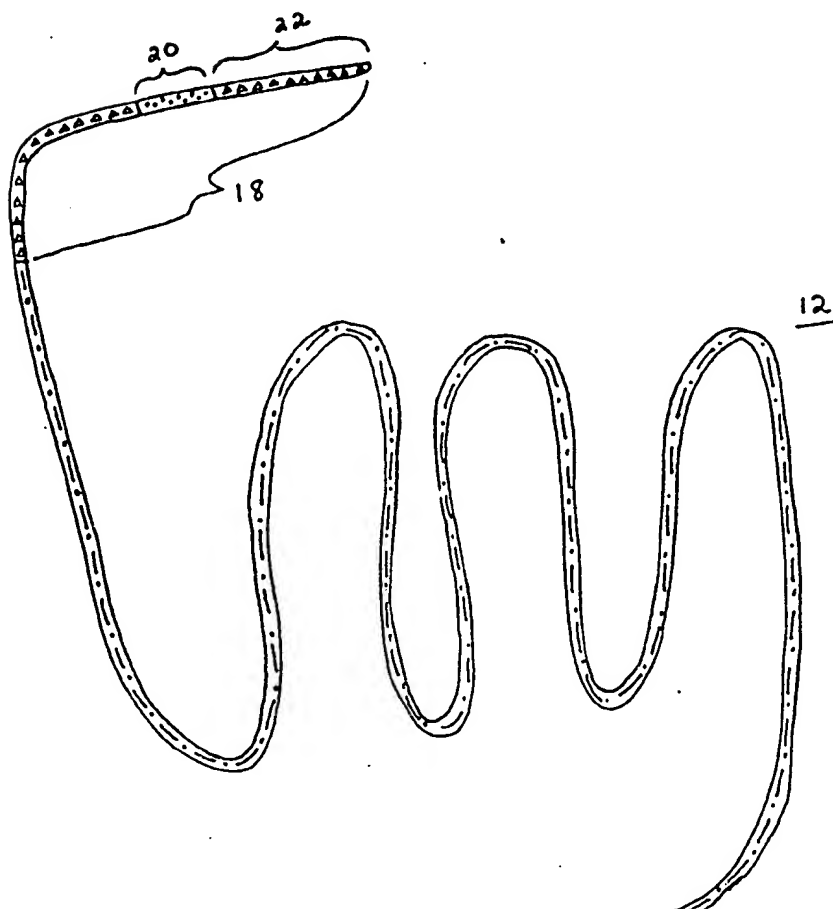
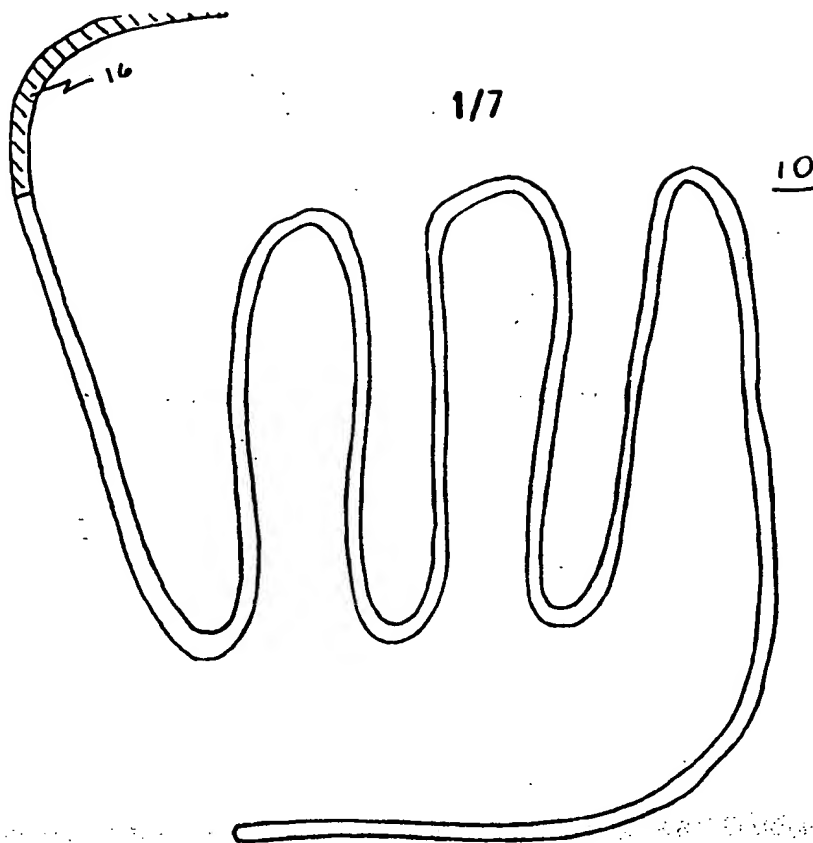
43. A cell comprising the G protein coupled receptor construct of claim 36.

44. The cell of claim 43 wherein the cell is a yeast cell.

45. An expression vector comprising the G protein coupled receptor construct of claim 36.

46. The expression vector of claim 45 wherein said expression vector is selected from the group consisting of a plasmid and a virus.

47. A cell comprising the expression vector of claim 45.



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Fig. 3

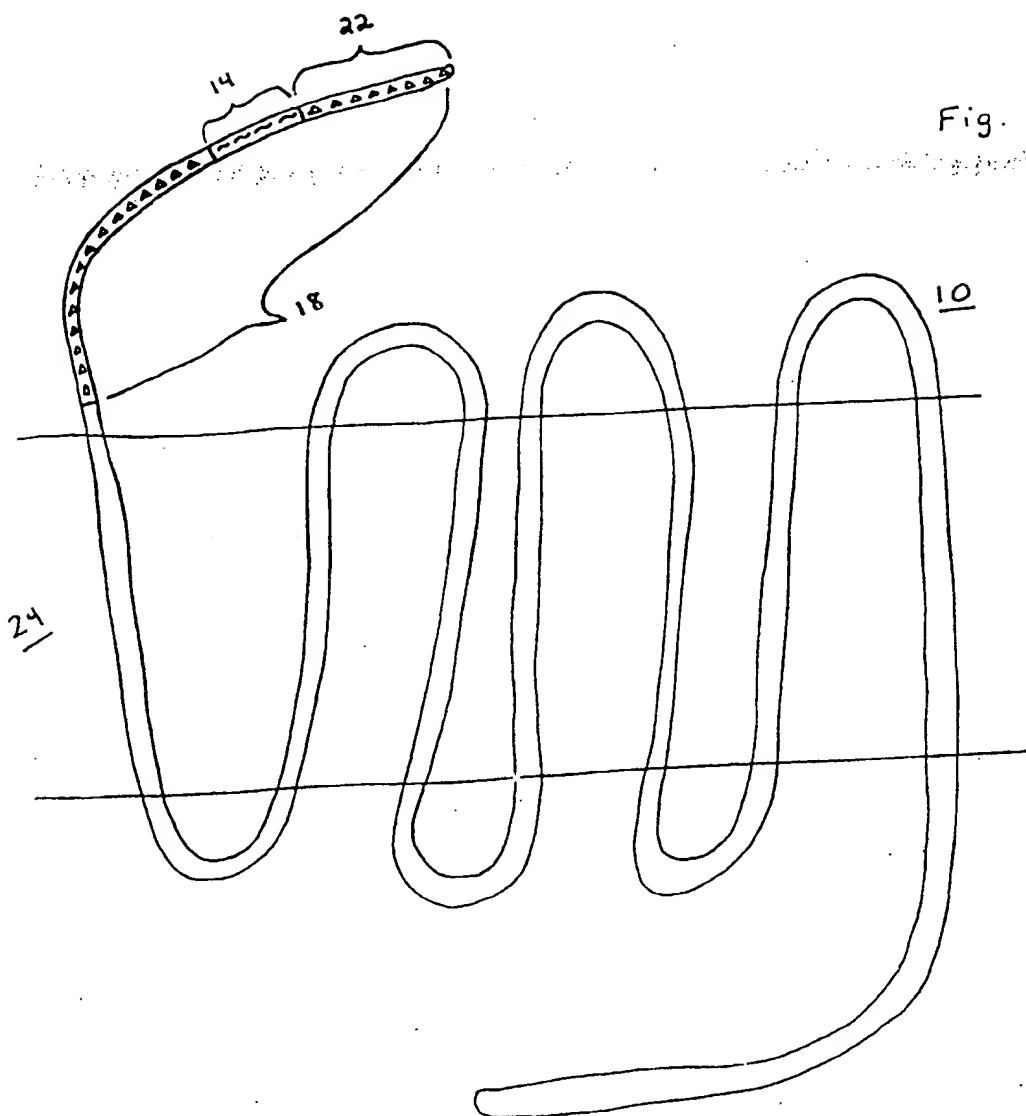
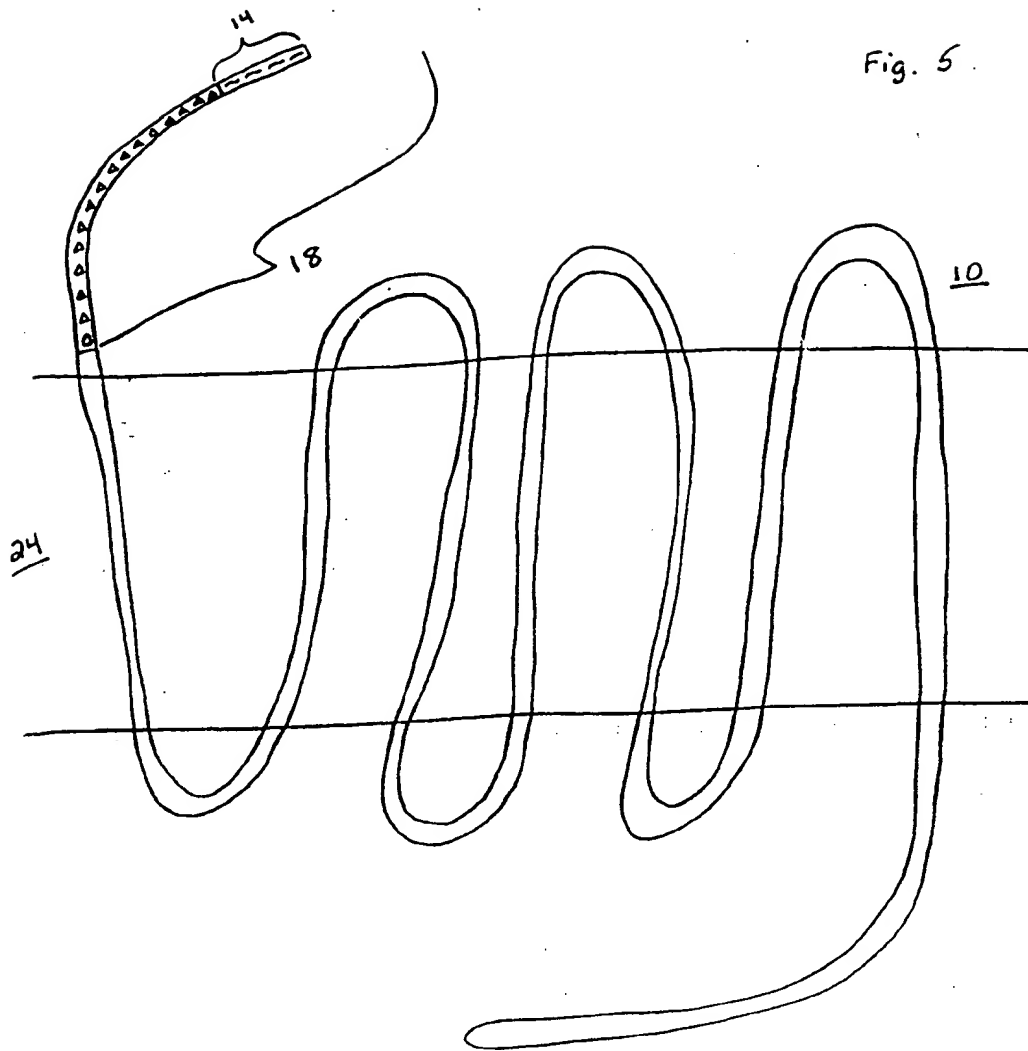


Fig. 4

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Fig. 5







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Fig. 7

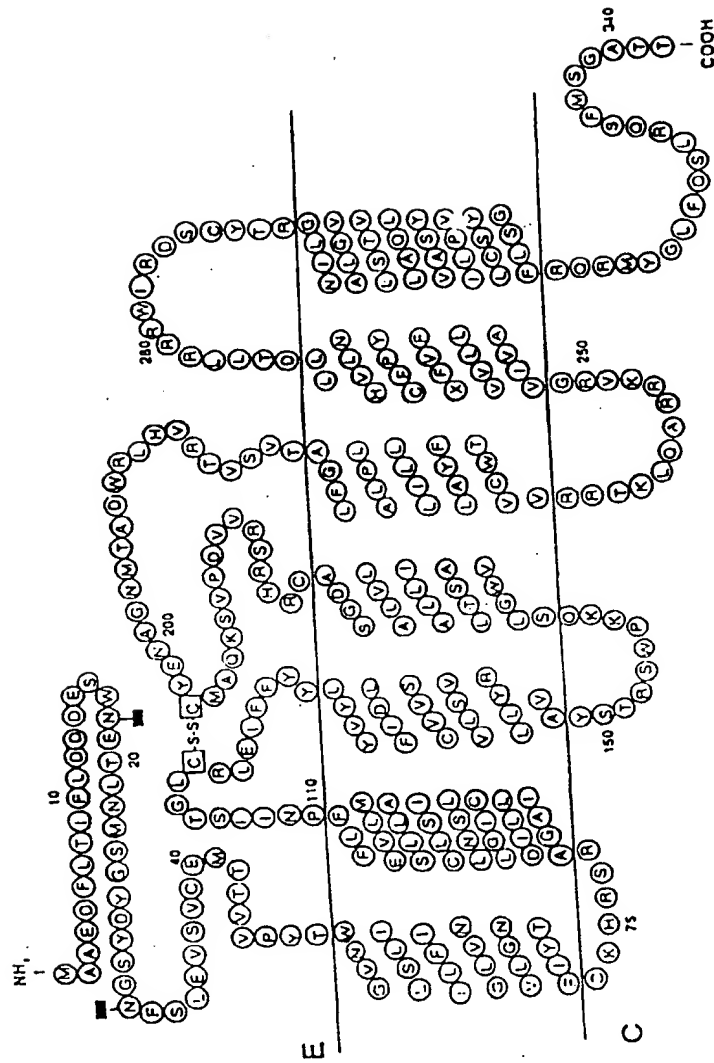
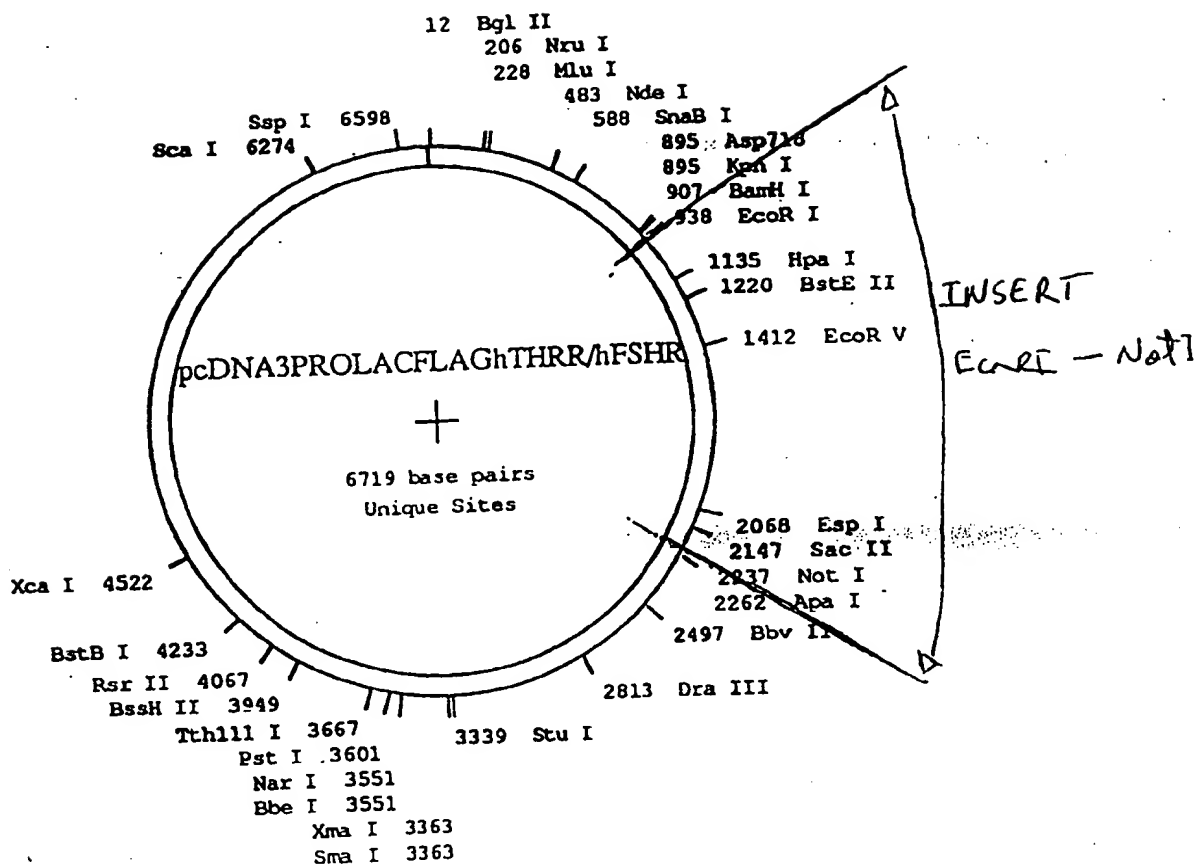




Fig. 9



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/02377

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.2, 69.1, 320, 325, 252.3, 254.11; 514/2; 530/300, 350; 536/23.1, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to claim No.
A	US 5,482,835 A (KING ET AL) 09 January 1996, entire document.	1-12, 14-22, 14-30, 32-47
A	PRICE ET AL. Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. Mol. Cell. Biol. November 1995. Vol. 15, No. 11, pages 6188-6195, especially abstract, Figure 1, and page 6189.	1-12, 14-22, 24-30, 32-47
A	JULIUS ET AL. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. Science. 29 July 1988. Vol. 241, pages 558-564, especially Figure 1.	1-31, 33-43, 45-47

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 APRIL 1998

Date of mailing of the international search report

29 MAY 1998

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/02377

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WRIGHT ET AL. Cloning strategies for peptide hormone receptors. Acta. Endocrinol. 10 March 1992. Vol. 125, pages 97-104, entire document.	1-47
A	CHEN ET AL. Tethered ligand library for discovery of peptide agonists. J. Biol. Chem. 06 October 1995. Vol. 270, No. 40, pages 23398-23401, especially Figure 1.	1-47